PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Timothy C. Thompson	Group Art Unit: 1642
Serial No.: 10/690,713	Examiner: Lei Yao
Filed: October 22, 2003	Atty. Dkt. No.: PRO025/4-9CON2US
For: METHODS FOR THE TREATMENT OF NEOPLASTIC DISORDERS WITH ANTI-CAVEOLIN AGENTS	Confirmation No. 9759

MAIL STOP APPEAL BRIEF - PATENTS
Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Sir:

The present notice of appeal and appeal brief are filed in response to a Final Official Action of December 4, 2007. A Notice of Appeal was filed on May 5, 2008, making the due date for this brief July 5, 2008. The Commissioner is requested to consider this a petition for a four month extension of time. Authorization to charge the amount of $1,135.00, including $270.00 for filing a brief in support of appeal according to 37 CFR 41.20(b)(2) and $865.00 for an extension of time of four months is enclosed herewith. If the authorization is inadvertently omitted, or should any additional fees be required for any reason relating to the enclosed materials, or should an overpayment be included herein, the Commissioner is authorized to deduct or credit said fees from or to Vinson & Elkins L.L.P. Deposit Account No. 22-0365/PRO025/4-9CONUS/67000.

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BRIEF ON APPEAL

I. REAL PARTY IN INTEREST

The real parties in interest are Timothy C. Thompson, Baylor College of Medicine and Progression Therapeutics, Inc.

II. RELATED APPEALS AND INTERFERENCES

Related US Application Serial No. 11/038285 is on appeal. An appeal brief has been filed by no decision has issued.

III. STATUS OF CLAIMS

Original Claims 1-25 were canceled in a preliminary amendment without prejudice. Claims 40-105 were canceled in response to a restriction requirement as drawn to non-elected inventions. Claims 32 and 34 were canceled in response to the first Office Action on the merits. Claims 26-31, 33, and 35-39 are rejected. The Final Rejection of Claims 26-31, 33, and 35-39 is the subject of the present appeal.

IV. STATUS OF AMENDMENTS

All amendments have been entered and considered by the Examiner.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The invention of independent claim 26 is described as a method of treating a metastatic disorder, such as metastatic prostate or breast cancer by administering to a

patient an effective amount of an anti-caveolin antibody [0019]. The antibody can be reactive against caveolin or the scaffolding domain of caveolin [0019].

The progression of neoplastic disease or disorders from normal to hyperplasia, early adenoma, early carcinoma and finally to a metastatic tumor is described in [0031]. It is an aspect of the claimed invention to inhibit or block this progression by inhibiting or blocking the activity of the caveolin protein, which is shown in the application to be directly related to the progression to metastatic disease. For example, the data in table 1 [0115] shows an increase in caveolin protein expression in metastatic prostate tumors than in primary tumors.

The invention of claim 35 is a method for treating a neoplastic disease of the prostate comprising administering to a subject in need thereof an anti-caveolin agent in conjunction with androgen ablation therapy. The Specification teaches that tumors produced by the antisense caveolin clones significantly regressed in response to surgical castration in vivo and that reduction of caveolin levels not only suppresses metastatic activity buy also restores androgen sensitivity [0079]. This is shown by the use of caveolin anti-sense clones that acquire hormone sensitivity [0081]. Treatment of prostate cancer in conjunction with androgen ablation is described at least at [0132].

VI. GROUND OF REJECTION TO BE REVIEWED

1. Are claims 26-31, 33, and 35-39 unpatentable under 35 USC §112, 1st paragraph for lack of enablement ?

VII. ARGUMENT

Claims 26-31, 33 and 35-39 are fully enabled by the Specification.

The Specification includes more than adequate support to teach one of skill in the

art how to practice the claimed inventions. The Specification teaches, for example, (i) caveolin is increased in metastatic prostate cancer [0080], (ii) caveolin is increased in androgen insensitive cancer [0117] and (iii) inhibition of caveolin restores androgen sensitivity when combined with castration therapy *in vivo* (Fig. 1).

Caveolin expression is increased in human metastatic prostate as compared to primary tumor or normal prostate tissue.

The Specification states in [0017] that caveolin expression increases in metastatic human prostate cells as compared to primary tumors and agents, and blocking the activity of caveolin in metastatic cells or cells predisposed to metastasis would be useful in treatment of human prostate tumors. A comparison of primary and metastatic tissues is shown in Table 1, Example 1 of the Specification. Furthermore, the Specification at [0080] incorporates Yang et al., submitted to the Examiner as Ref. HHH in an Information Disclosure Statement on October 22, 2003, and attached as Exhibit 4. The Yang reference was published by the inventor after the priority date of the present application and shows the increased expression of caveolin in metastatic prostate cells relative to cells from primary tumors, Fig. 1C.

Yang also reports immunhistochemical staining of mouse normal, primary tumor, and metastatic tissues to detect caveolin levels. The reference states:

> To validate the in vitro studies using cell lines, a series of immunohistochemical studies were undertaken to assess the pattern and amount of caveolin expression in tissue specimens of both primary and metastatic prostate carcinoma... The results demonstrated only minimal caveolin expression in normal mouse prostate epithelial cells within the prostate gland; however abundant caveolin staining was observed in smooth muscle cells, which uniformly surround mouse prostate acini as well as endothelial cells in the stromal compartment (Fig. 3A). A diffuse, increased accumulation of caveolin was seen in primary prostate cancer (Fig. 3B), and in the corresponding metastatic cancer cells

> within the mesentery, higher levels of caveolin appearing as a granular pattern localized near the plasma membrane were seen (Fig. 3C). In normal human prostate, as in the mouse, accumulation was seen in smooth muscle cells as well as endothelial cells with minimal or no staining of ductal or acinar epithelial cells (Fig. 3D) In primary prostate cancer, detectable accumulation of caveolin in malignant cells was occasionally observed (Fig. 3E), whereas in metastatic cancer within lymph nodes, an obvious granular accumulation of caveolin was seen in the carcinoma cells (Fig. 3F). Yang, pg 1876)

The Specfication thus establishes as association of increased caveolin expression from normal to primary tumor to metastatic neoplastic disease in the human prostate.

The Specification contains an enabling description of treatment of human prostate cancer.

The Specification further describes treating prostate cancer and metastatic prostate disorders by administering an anti-caveolin antibody, at least at paragraphs [0019] [0077] [0079] [0080] [0087] [0090] and [0095], all of which teach one of skill in the art that suppression of caveolin activity is useful in the treatment of metastatic prostate cancer and prostate neoplasia with potential to progress to become metastatic. Although the *in vivo* data were obtained by genetic suppression of caveolin, either by antisense or knockout constructs, one of skill in the art clearly understands that the suppression of caveolin activity can also be achieved by the use of antibody therapy as described. The use of anti-caveolin antibodies in inhibition of metastasis in prostate disease is thus fully enabled.

None of the cited prior art refutes the Specification's teaching that inhibition of caveolin with antibody therapy is an effective treatment for prostate cancer.

The Final Action argues that the Specification is not enabling because treating tumors with antibodies is unpredictable and that there is not "convinced evidence that

caveolin is directly related with tumorgenesis and is useful therapeutic target." On the contrary, the current state of the art has greatly reduced the relevancy of the prior art relied upon by the Action. Several antibodies have been approved for cancer treatment, and studies show that caveolin is directly linked to prostate cancer progression.

Monoclonal antibodies are increasingly recognized as important agents for the treatment of cancer.

The Action relies upon previously cited articles suggesting the "unpredictability of treating tumors with antibodies." The Action relies on the Jain, Dillman, Weiner, and Nelson references to support this rejection. None of the cited art, either alone or in combination refutes the description in the Specification that inhibition of caveolin with antibody therapy would be beneficial in the treatment of neoplastic disease of the prostate, and more particularly that such treatment would inhibit metastasis as in claim 26, or restore androgen dependence as in claim 35.

The references relied on by the Action do not refute the enablement of Appellant's asserted utility.

Jain describes some obstacles to systemic delivery of cytotoxic chemicals to solid tumors at concentrations sufficient to eliminate the malignancies from the body. Such a discussion is not relevant to the claimed inventions, which are not necessarily directed to total elimination of a primary tumor. Jain, for example, does not discuss the possibility of treating a prostatic disease by inhibiting the function of a protein such as caveolin, or inhibiting progression of the disease to a metastatic state, but focuses the discussion on achieving lethal concentrations of a cytotoxic agent throughout a solid tumor.

The Action also cites Dillman, a review article about treatment with monoclonal antibodies that was written in 1989, when monoclonal antibody therapy was a relatively

new technology. Even the Dillman abstract states that monoclonal antibodies are a promising therapy but that their general use will be delayed for several years. It has now been sixteen years since Dillman made that statement. One of skill in the art would not look to such a dated review article when evaluating a therapeutic approach and would find nothing in the Dillman article that has any relevance to the present claims.

The Action also points to alleged major obstacles to antibody therapy in the Weiner article. The abstract of Weiner states, however, that monoclonal based therapeutics have shown efficacy in clinical trials, and further states that these exciting results justify the enthusiasm for continued efforts to refine the existing approaches. The Weiner reference thus argues for the enablement of the claims in spite of certain obstacles that Weiner states are not reason for discouragement. Turning to the more recent of the Dillman articles, the section entitled *Monoclonal Antibodies as Biologic Response Modifiers* beginning on page 1505 may have some relevance to the claimed invention, since inhibition of caveolin activity can be considered a biologic response treatment, based on the data in the Specification in which the caveolin is genetically suppressed. This appears more relevant than the short discussion of treating prostate cancer with anti-PSA or anti-prostatic acid phsophatase. This review, written in 1994 indicates that such therapies including antibodies against receptors such as Her2-neu, transferring and epidermal growth factor were in very early stages of development thirteen years ago.

Appellants submit that none of the references discussed above are relevant to the enablement of the present claims, first because they are discussing a technology that has advanced significantly since their publication dates, and second because none of the references, even at those early dates, dispute the efficacy of antibody therapy as suggested by the Action.

The Action appears to mischaracterize Nelson, taking a single phrase out of context to state that Nelson teaches that reduced caveolin expression might be considered ineffective. When the statement is fully considered, as it would be by one of skill in the art, to learn what the author actually meant, the opposite conclusion is reached.

> Although the progression of caveolin-depleted tumors is less than that of control, the tumors still progress. If a similar caveolin targeting strategy were successfully applied in humans using tumor growth as the endpoint, the therapy would be considered ineffective because the tumors would continue to grow, albeit more slowly. But prostate tumors are characterized by low rates of proliferation and apoptosis; **therefore, any therapy that prolongs survival deserves consideration.** This seemingly axiomatic concept has not enjoyed wide acceptance by those demanding evidence of cytotoxicity as the final measure of efficacy. Until a cure for prostate cancer is found, **new therapies producing disease stabilization should not be dismissed.** (Emphasis added) Nelson, pg 1011

The references relied upon by the Action in no way provide support for a blanket rejection of all treatments of cancer with antibodies. Furthermore, one of skill in the art would not rely upon such references to determine whether the Specification was enabled the use of anti-caveolin antibodies for the treatment of prostate cancer.

Previously cited publications support the enablement of the claimed inventions.

Appellant submits that recent reviews are more accurate reflections of the state of the art of antibody therapies for cancer, and are indicative of what one of skill would understand upon reading the Specification.

Strome, et al., (submitted as Exhibit 1 in response to Final Office Action) review six monocolonal antibodies that have been approved by the U.S. Food and Drug

Administration for use in cancer therapy. *A Mechanistic Perspective of Monoclonal Antibodies in Cancer Therapy Beyond Target-Related Effects*, The Oncologist, 12:1084-95, (2007) (*see* Table 1 for list of six antibodies approved by the FDA) (attached as Exhibit 1). Strome et al. conclude:

> "mAbs [Monoclonal antibodies] represent an important advance in the treatment of certain hematologic malignancies and solid tumors. Unlike many small molecules, mAbs offer unique target specificity. **The field has evolved rapidly in recent years, and now it is much easier to create mAbs against a variety of targets of potential relevance to tumor growth and survival.**" Strome at 1092, emphasis added.

Likewise, Sharkey, et al., (submitted with response to Final Office Action as Exhibit 2) similarly describe the state of antibody cancer treatments:

> "Immunotherapy of cancer has been explored for over a century, but it is only in the last decade that various antibody-based products have been introduced into the management of patients with diverse cancers. **At present, this is one of the most active areas of clinical research, with eight therapeutic products already approved in oncology. Antibodies against tumor-associated markers have been a part of medical practice in immunohistology and *in vitro* immunoassays for several decades, have even been used as radioconjugates in diagnostic imagining, and are now becoming increasingly recognized as important biological agents for the detection and treatment of cancer.**" *Targeted Therapy of Cancer: New Prospects for Antibodies and Immunoconjugates*, CA: A Cancer Journal for Clinicians, 56:226-43, abstract (2006) (attached as Exhibit 2)

These articles are but two examples of many publications describing the development and use of antibodies in cancer treatment. Notably, at least six antibodies have endured and survived the rigorous and thorough testing process required for Food and Drug Administration approval. Thus, in light of these reports on the state of the art, one of skill in the art would immediately recognize that monoclonal antibodies are useful

in the treatment of cancer and would recognize the Specification as enabling for such treatment.

The fact that the FDA has approved at least six antibodies for the treatment of various cancers refutes the Action's general argument that treating cancer with antibodies is unduly "unpredictable." There is always an element of unpredictability in the development of biological therapies because biological systems are complex and interrelated, but such unpredictability does not present a rational basis for rejecting an otherwise enabled claim. To the contrary, as evidenced by the FDA's approval, antibodies have been shown to be predictably therapeutic in treating cancer.

Studies confirm that caveolin is directly related to tumorgenesis.

The Final Action also argues: "the prior art does not provide convinced evidence that caveolin is directly related with tumorgenesis and is useful therapeutic target . . . . the prior art has not settled the question of the biological function of caveolin in neoplastic disorder comprising prostate or breast cancer."

Appellant submits as Exhibit 3 a peer-reviewed, published article co-authored by the inventor, Timothy C. Thompson, which confirms the Specification's disclosure with respect to the role of caveolin in prostate cancer as taught in the Specification. Tahir, et al., *Tumor Cell-Secreted Caveolin-1 Has Proangiogenic Activities in Prostate Cancer*, Cancer Research, 68:731-39 (February 1, 2008) (submitted with response to Final Office Action as Exhibit 3). In this article, the authors "examined the association between cav-1 expression and prostate tumor-associated angiogenesis" by generating an LNCaP (human prostate adenocarcinoma) tet-on cav-1 cell line (referred to as LNTB25cav) in which cav-1 was regulated by doxycycline. Tahir at 6. In one assay, LNTB25cav tumors were established as subcutaneous xenografts in nude mice. *Id.* at 7. Tumor-bearing mice were

treated with either doxycycline or a control solution. *Id.* The doxycycline induced cav-1 expression in the tumors and resulted in significantly greater tumor volumes. *Id.* at 7 and Fig. 6. These data confirm that caveolin expression contributes to tumor growth. In a second assay, the LNTB25cav cells were injected into the tail veins of nude mice to study lung metastases. The test group was treated with cav-1 inducing doxycycline for 42 days. When compared to the control group, "the number and frequency of lung metastases in doxycycline-treated [cav-1 induced] animals significantly exceeded results in the control group . . . and their average size was clearly larger in doxycycline-treated mice." *Id.* at 7 and Fig. 6. These data confirm that caveolin expression contributes to tumorgenesis. Thus, taken together, these data confirm the Specification's disclosure that caveolin is directly related to tumor growth and size.

In light of these comments and submitted evidence, Appellants assert that all the pending claims are enabled by the Specification and respectfully request that the Board overturn the rejections of all appealed claims.

Claims 35-39, drawn to treatment in conjunction with androgen ablation therapy are separately patentable, and are fully enabled by the Specification.

The Specification demonstrates that inhibition of caveolin restores androgen sensitivity to prostate cancer.

It is an important aspect of the disclosure that inhibiting expression or activity of caveolin restores androgen sensitivity to prostate cancer. It is well known that prostate cancer is androgen dependent, or in other words, prostate cancer will not grow in the absence of androgen, and a primary treatment for prostate cancer includes androgen deprivation (Spec. at [0013]) Certain tumors, however, become androgen insensitive and no longer require androgen to grow. When this occurs, the tumor no longer responds to

one of the most effective available treatment options. Restoring androgen sensitivity by concurrently suppressing caveolin and androgen is an important and novel contribution to the art.

This combination therapy and restoring androgen sensitivity are described in the Specification at least at paragraphs [0021] [0079] [0081] [0085] and [0117], and in Example 2, starting at [0116]. As stated at [0079]

> "Surprisingly it has been discovered that tumors produced by the antisense caveolin clones significantly regressed in response to surgical castration in vivo. Eleven days following androgen ablation, tumors derived from three independent antisense clones regressed by approximately 30% relative to the wet weights-produced in either vector-control clone or parental clones which did not respond to castration therapy under the same conditions. The antisense caveolin tumors that responded to castration therapy also demonstrated significantly increased levels of apoptosis relative to either vector-control cones or parental cell lines. Therefore, the data indicates that reduction of caveolin levels not only suppresses metastatic activity buy also restores androgen sensitivity.

The treatment of prostate cancer by suppressing caveolin with an anti-caveolin antibody in conjunction with reducing androgen levels, therefore, is fully enabled by the Specification.

In light of the preceding discussion and evidence, Appellants respectfully request the Board to overturn the rejection of claims 35-39 for lack of enablement.

VIII. Conclusion

Appellants submit that all the Examiner's rejections and objections are overcome in light of the preceding arguments and evidence and Appellants request that all rejections be overturned and the pending claims allowed without further amendment or prosecution.

IX. CLAIMS APPENDIX

1-25 (canceled)

26. (previously presented) A method for treating a subject having a prostatic neoplastic disorder comprising administering to the subject a composition comprising an anti-caveolin antibody wherein the antibody is effective to inhibit metastasis in the neoplastic disorder.

27. (previously presented) The method of claim 26, wherein the neoplastic disorder is a displasia.

28. (previously presented) The method of claim 26, wherein the neoplastic disorder is hyperplasia, dysplasia or a hypertrophy.

29. (previously presented) The method of claim 26, wherein the neoplastic disorder is benign enlargement of the prostate, nodular hyperplasia or benign prostatic hypertrophy.

30. (previously presented) The method of claim 26, wherein the neoplastic disorder is a malignancy.

31. (previously presented) The method of claim 26, wherein the neoplastic disorder is hormone responsive.

32. (canceled)

33. (previously presented) The method of claim 26, wherein the neoplastic disorder is prostate cancer.

34. (canceled)

35. (previously presented) A method for treating a neoplastic disease of the prostate comprising administering to a subject in need thereof an anti-caveolin agent in conjunction with androgen ablation therapy.

36. (previously presented) The method of claim 35, wherein the anti-caveolin agent is an anti-caveolin antibody.

37. (previously presented) The method of claim 35, wherein the antibody is a monoclonal antibody.

38. (previously presented) The method of claim 35, wherein the antibody is a polyclonal antibody.

39. (previously presented) The method of claim 35, wherein the androgen ablation therapy comprises administration of a composition comprising an anti-androgen antibody to the subject.

40. - 105. (canceled)

X. EVIDENCE APPENDIX

1. Strome et al., A Mechanistic Perspective of Monoclonal Antibodies in Cancer Therapy Beyond Target-Related Effects, *Oncologist* 2007; 12;1084-1095

2. Sharkey and Goldenberg, Targeted Therapy of Cancer: New Prospects for Antibodies and Immunoconjugates, *CA A Cancer journal for Clinicians*, 2006;56;226-243

3. Tahir et al., Tumor Cell-Secreted Caveolin-1 Has Proangiogenic Activities in Prostate Cancer, *Cancer Research*; 2008;68;1-9

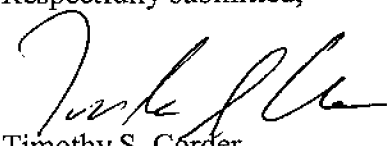
4. Yang et al., Elevated Expression of Caveolin Is Associated with Prostate and Breast Cancer, *Clinical Cancer Research*, 1998; 4;1873-1880.

XI. RELATED PROCEEDINGS APPENDIX

US Application Serial No. 11/038285

No decision has issued.

Respectfully submitted,



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The Oncologist®

A Mechanistic Perspective of Monoclonal Antibodies in Cancer Therapy Beyond Target-Related Effects

Scott E. Strome, Edward A. Sausville and Dean Mann

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EXHIBIT 1

The Oncologist®
Clinical Pharmacology

A Mechanistic Perspective of Monoclonal Antibodies in Cancer Therapy Beyond Target-Related Effects

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Key Words. Cancer • Monoclonal antibodies • Antibody-dependent cellular cytotoxicity • Complement-dependent cytotoxicity

Disclosure: S.E.S. owns stock in Gliknik, has acted as a consultant for Accuitive Medical Ventures, has performed contract work for GTC Biotherapeutics, and receives licensing revenue from IP agreements between Mayo Clinic and various third parties (as an inventor).

LEARNING OBJECTIVES

After completing this course, the reader will be able to:

1. Describe the relationship between antibody structure and effector function, and identify strategies for modifying antibody structure to enhance these functions.
2. Explain how the efficacy of monoclonal antibodies in cancer therapy may occur via antibody- as well as target-related mechanisms.
3. Discuss how the ability of monoclonal antibodies to activate immune-mediated effector functions differs across antibody isotypes.

ABSTRACT

Several monoclonal antibodies are now in clinical use for cancer therapy, and many others are currently undergoing clinical evaluation. These agents offer unique specificity against key molecular targets on tumor cells or in the tumor microenvironment. The clinical efficacy of monoclonal antibodies is generally attributed to target-specific mechanisms resulting from neutralizing or inhibiting a growth factor or receptor that drives cell proliferation and tumor growth. Several targets, including CD20, human epidermal growth factor receptor 2, vascular endothelial growth factor, and epidermal growth factor receptor, have been validated in specific malignancies on the basis of monoclonal antibody efficacy. However, monoclonal antibodies also have the potential to activate immune-mediated effector functions, including antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity. These functions result from interactions involving the Fc domain of the antibody, and, consequently, may vary by antibody, isotype, and Fc modification, such as changes in glycosylation. Accordingly, all monoclonal antibodies directed against a given target should not be considered equivalent in their ability to stimulate immune-mediated effector functions. *The Oncologist* 2007;12:1084–1095

Correspondence: Scott E. Strome, M.D., F.A.C.S., Department of Otorhinolaryngology—Head and Neck Surgery, 16 South Eutaw Street, Suite 500, Baltimore, Maryland 21201-1619, USA. Telephone: 410-328-2378; Fax: 410-328-6192; e-mail: sstrome@smail.umaryland.edu Received March 2, 2007; accepted for publication June 27, 2007. ©AlphaMed Press 1083-7159/2007/$30.00/0 doi: 10.1634/theoncologist.12-9-1084

Table 1. Unconjugated monoclonal antibodies currently approved by the U.S. Food and Drug Administration for use in cancer therapy [2, 5]

Antigenic target	Monoclonal antibody	Year approved	Antibody construct	Isotype	Cancer indication
CD20	Rituximab	1997	Chimeric	IgG_1	Non-Hodgkin's lymphoma
HER-2	Trastuzumab	1998	Humanized	IgG_1	Breast cancer
CD52	Alemtuzumab	2001	Humanized	IgG_1	Chronic lymphocytic leukemia
VEGF	Bevacizumab	2004	Humanized	IgG_1	Colorectal cancer
EGFR	Cetuximab	2004	Chimeric	IgG_1	Colorectal cancer
EGFR	Panitumumab	2006	Human	IgG_2	Colorectal cancer

Several additional monoclonal antibodies are immunoconjugates that either deliver radioisotopes or cytotoxic agents to tumor cells. Ibritumomab tiuxetan and tositumomab are anti-CD20 conjugates that deliver ^{90}Y and ^{131}I, respectively, to tumor cells in non-Hodgkin's lymphoma, whereas gemtuzumab ozogamicin is an anti-CD33 conjugate that delivers a cytotoxic calicheamicin derivative to acute myelogenous leukemia cells [2].
Abbreviations: EGFR, epidermal growth factor receptor; HER-2, human epidermal growth factor receptor 2; VEGF, vascular endothelial growth factor.

Introduction

Monoclonal antibodies (mAbs) became a therapeutic possibility for cancer with the development of hybridoma technology by Kohler and Milstein in 1975 [1]. This technology allowed the immortalization of antibody-producing cells derived from immunized mice and the subsequent selection of single-cell clones for the production of antibodies with high affinity and single specificity for an antigenic target. Early studies showed that murine mAbs directed against tumor antigens were effective in animal models, but translation of these findings into a clinical setting proved to be problematic [2]. The poor performance of mAbs in these early studies was attributed to short antibody half-life, immunogenicity of the murine protein in the human host, and depressed immune-mediated effector functions [3]. Importantly, it raised the question of whether mAbs directed against tumor antigens could elicit a sufficient immune response to promote clinically meaningful tumor regression.

Many of these early limitations were overcome by generating chimeric or humanized mAbs [2, 3]. A chimeric antibody contains the murine variable region (the part of the antibody that specifically recognizes its antigenic target) fused to constant domains of a human antibody backbone [4]. In comparison, a humanized antibody contains the murine sequence of only those sections of the variable domain that actually interact with the antigenic target. These so-called complementarity-determining regions (CDRs) are grafted onto a human antibody. Finally, fully human antibodies contain no murine sequences. Because most or all of the murine sequence has been replaced, the chimeric, humanized, and human antibodies are less immunogenic and may have longer half-lives because of a slower clearance [2].

Within the body, antibodies identify and label alien, potentially harmful particles, an initial step in the destruction of pathogens or abnormal cells. Subsequently, other components of the immune system attack and destroy the targets tagged by antibodies.

For therapeutic purposes, it was recognized that high-specificity binding by antibodies could neutralize membrane proteins regulating tumor growth. By blocking these growth factor receptors, antibodies could promote apoptosis or arrest growth of tumor cells merely by binding their target, thereby obviating the need to stimulate immune effector functions. These advances led to the development and approval of the first two mAbs for use in cancer therapy: rituximab (Rituxan®; Genentech, Inc., South San Francisco, CA) for non-Hodgkin's lymphoma in 1997 and trastuzumab (Herceptin®; Genentech, Inc., South San Francisco, CA) for breast cancer in 1998. Currently, at least six unconjugated mAbs are now approved by the U.S. Food and Drug Administration for use in cancer therapy (Table 1) [2, 5]. Several other approved mAbs are immunoconjugates, which are designed to deliver radioisotopes or cytotoxic agents specifically to tumor cells.

Target-specific mechanisms likely account for much of the efficacy of mAbs in cancer therapy. However, sufficient data exist now to suggest that other antibody-related mechanisms may contribute significantly to the activity of some mAbs. Therefore, mAbs directed against the same antigenic target may, in theory, differ in their clinical profile depending on whether or not they effectively activate immune-mediated effector functions. This article considers evidence on the role of immune mechanisms, specifically antibody-dependent cell-mediated cytotoxicity (ADCC) and comple-

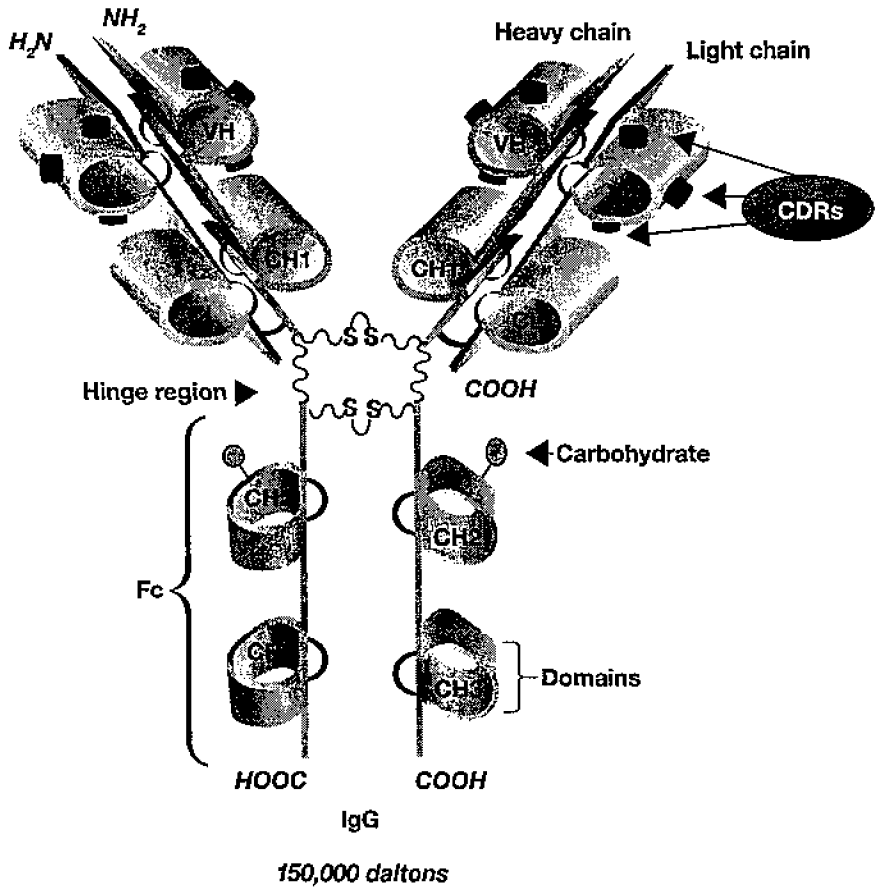

Figure 1. Schematic of IgG antibody structure. Abbreviation: CDRs, complementarity-determining regions. From Sharkey RM, Goldenberg DM. Targeted therapy of cancer: New prospects for antibodies and immunoconjugates. CA Cancer J Clin 2006;56: 226–243, with permission.

ment-dependent cytotoxicity (CDC), in the action of mAbs in cancer therapy.

Antibody Structure and Effector Function

Antibody Structure and Isotypes

Antibodies are heterodimers consisting of two light chains and two heavy chains, in which each light chain is attached to a heavy chain by a disulfide bond, and the heavy chains are attached to each other by multiple disulfide bridges (Fig. 1) [6, 7]. The amino terminus of each light and heavy chain contains the variable region, which differs in amino acid sequence across antibodies. The unique specificity of antibodies depends on the amino acid sequence of the CDRs located within the variable region. Together, the CDRs on both the light and heavy chains form a unique structural conformation that represents the antigen-binding site of the antibody. The rest of the antibody molecule, known as the constant region, shows relatively few differences in amino acid sequence. On treatment with the enzyme papain, antibodies are degraded into two identical Fab fragments, each containing the antigen-binding site, and an Fc fragment without antigen-binding activity [7]. It is through the antigen-binding site that an antibody recognizes the antigenic epitope of its target, thus conveying its unique specificity. Whereas the antigen-binding site determines the antibody specificity, it is the Fc region that binds to effector cells or complement to trigger immune-mediated effector functions [6, 8].

Amino acid sequencing shows that the constant region of light chains can be classified into two basic types, termed κ and λ. The κ type accounts for a large majority of murine light chains but only 60% of light chains in human antibodies. Most of the engineered antibodies for therapeutic purposes use κ light chains. There are also several basic types of heavy chains, which are classified according to the amino acid sequence pattern of their constant regions. These are designated as γ, μ, α, δ, and ε, and correspond to IgG, IgM, IgA, IgD, and IgE, respectively. Each type of heavy chain can be combined with either a κ or λ light chain. The γ heavy chain can be further classified on the basis of minor differences in amino acid sequence into four

Table 2. Rank order of binding affinity of IgG isotypes for FcγR subclasses [15]

FcγR subclass	Polymorphism	Rank order of binding
FcγRI		$IgG_3 > IgG_1 > IgG_4$ [tmt] IgG_2
FcγRIIa	*131R 131H*	$IgG_3 > IgG_1$ [tmt] $IgG_2 = IgG_4$ $IgG_3 > IgG_1 = IgG_2$ [tmt] IgG_4
FcγRIIb		$IgG_3 > IgG_1 > IgG_4 \gg IgG_2$
FcγRIIc		Not determined
FcγRIIIa	*158V* or *158F*	$IgG_1 = IgG_3$ [tmt] $IgG_2 = IgG_4$
FcγRIIIb	*NA1* or *NA2*	$IgG_1 = IgG_3$ [tmt] $IgG_2 = IgG4$

Abbreviations: FcγR, immunoglobulin G fragment C receptor.

isotypes, termed γ1, γ2, γ3, and γ4, which in turn give rise to four IgG isotypes, termed IgG_1, IgG_2, IgG_3, and IgG_4, respectively. These isotypes are distinguished by the size of the hinge region separating the variable and constant regions, and by the number and position of the disulfide bridges linking the two heavy chains [7]. These relatively small changes in structure may substantially impact the biological activity of antibody isotypes [9]. Within each isotype, the overall structure and function of immunoglobulins may also be determined by glycosylation status and by allelic variations. It has been shown that several glycosylation sites are critical for the full functionality of antibodies. Ig variants engineered to lack glycosylation cannot bind their Fc receptors (FcRs), while, inversely, function can be improved by manipulating the composition of the oligosaccharide chains attached to the Ig backbone [10–14]. The effect of allelic variants on immune functionality is described below, as it has been crucial to assess the contribution of these functions to overall therapeutic efficacy.

Immune-Mediated Effector Functions

Immune-mediated effector functions include two major mechanisms: ADCC and CDC. Both of them are mediated by the constant region of the immunoglobulin protein. The antibody Fc domain is, therefore, the portion that defines interactions with immune effector mechanisms.

ADCC

In ADCC, an IgG antibody first binds via its antigen-binding site to its target on tumor cells, and then the Fc portion is recognized by specific Fcγ receptors (FcγR) on effector cells [3]. In humans, the FcγR expressed on leukocytes include high-affinity FcγRI (which binds to monomeric IgG and tends to be occupied by plasma IgG) and low-affinity FcγRII and FcγRIII (which bind IgG aggregates or immunocomplexes), each having several isoforms with differing cellular localization.

The intracellular structures of FcγRIs and FcγRIIIs contain activation domains that can stimulate immune cells via Src-family protein tyrosine kinases. Fc binding in the context of Fab ligation results in FcγR crosslinking, thereby activating intracellular signaling and ultimately stimulating their effector functions. However, Fc binding to FcγIIb, which is expressed by B cells, macrophages, and monocytes, induces an inhibitory signal that may serve to regulate effector functions. Natural killer (NK) cells are the principal effectors of ADCC; they express FcγRIIc and FCγRIIIa. The role of FcγRIIc activation in NK cells is unclear, but activation of FcγRIIIa induces ADCC and cytokine production [15]. ADCC is mediated by the release of cytotoxic granules, such as perforin, granulysin, and granzymes, whereas the release of cytokines and chemokines leads to inhibition of cell proliferation and angiogenesis. Macrophages also express FcγRIIa and FcγRIIIa, and can induce phagocytosis of antibody-coated tumor cells as well as promote ADCC through release of proteases, reactive oxygen species, and cytokines [3].

The ability of mAbs to stimulate ADCC depends on their isotype. IgG_1 and IgG_3 antibodies bind very well to FcγRs, while IgG_4 and IgG_2 antibodies bind weakly (Table 2) [7]. Therefore, both IgG_1 and IgG_3 isotypes can provide a double-pronged therapeutic action: target-based and immune-based. IgG_3 antibodies, however, have a much shorter serum half-life (8 days versus 23 days for IgG_1s) probably due to different interactions between the two isotypes and the Fc neonatal receptor (FcnR) that regulates immunoglobulin homeostasis. This relatively short half-life makes the IgG_3 class suboptimal for therapeutic administration (except when short life poses an advantage; see immunoconjugates, below), and most mAbs currently available for cancer therapy are of the IgG_1 isotype (Table 1). These monoclonal IgG_1s allow for feasible administration and are most likely to promote ADCC, thus contributing an additional mechanism to their antitumor activity.

CDC

CDC is another immune-mediated effector function that depends on antibody isotype. IgG_3 followed by IgG_1 are the most effective isotypes for stimulating the classic complement cascade: both isotypes bind to C1q leading to formation of C3b on the surface of antibody-coated tumor cells near the site of complement activation [7]. IgG_2 antibodies are less efficient in activating the complement cascade, whereas IgG_4 is unable to do so [4]. The presence of C3b controls formation of the C5–C9 membrane attack complex (MAC) that can insert into the membrane to lyse tumor cells. However, the enzymatic activity of C3b and consequently MAC formation are regulated by a series of membrane proteins that are overexpressed on many tumor cells [2]. These include CD35 (complement receptor type I), CD46 (membrane cofactor protein), and CD55 (decay accelerating protein), which inactivate the enzymatic activity of C3b, and CD59, which inhibits MAC formation; these markers have been demonstrated to inhibit tumor cell lysis by complement mediated by a therapeutic IgG_1 [16]. Therefore, a substantial contribution of CDC to the antitumor activity of mAbs may be unlikely, given the presence of these negative regulators in tumor cells [3]. Nevertheless, although C3b is inactivated to iC3b, its presence on the tumor cell surface may be recognized by effectors expressing receptors for C3 fragments, and in turn leads to synergistic interactions with FcγR-mediated phagocytosis or ADCC [2, 17, 18].

Studying Immune Functions: Murine Models Versus Human Systems

Many of the studies that have shaped our understanding of immunologic functions have been carried out in murine models. It is worth considering, therefore, the substantial differences between the FcR system in humans and their murine counterparts. The FcR systems in mice and humans share a similar architecture, with a complex stimulatory/inhibitory receptor network, but the relative affinities of the stimulatory elements for each different isotype in mice do not match those in the human FcR family.

In mice, the FcγRI is also a high-affinity ADCC-stimulatory receptor, but it binds preferentially the IgG_2 isotype (in particular, the subisotype IgG_{2a}) and seems to have a limited role in the functions of other murine IgGs [19, 20]. The activity of the murine FcRγIII seems to be complementary to FcγRI, binding the isotypes IgG_1 and IgG_{2b} more robustly than the IgG_{2a} [21–23]. Finally, a third stimulatory receptor, FcγRIV, has high homology with the human FcγRIII and moderate affinity for the IgG_2 isotype (a and b), but does not bind IgG_1 [24].

Mechanism and Activity of Therapeutic mAbs

Target-Specific Mechanisms

Depending on the epitope against which an antibody is directed, antibody–antigen binding may neutralize circulating targets or cell surface receptors. Antibody binding to a receptor may prevent natural activation by ligands, or actually promote receptor activation. The epitope for antibody binding is very critical because some tumors may change surface proteins by post-translational modification, and consequently an antibody that recognizes a normal or unmodified antigen may no longer bind once the antigen has been modified. The currently available unconjugated mAbs are directed against molecular targets that are expressed on tumor cells or play an important role in the tumor microenvironment (Table 1).

Bevacizumab

Bevacizumab (Avastin®; Genentech, Inc., South San Francisco, CA) is a humanized IgG_1 mAb directed against vascular endothelial growth factor (VEGF). VEGF binds to VEGFR-1 and VEGFR-2 receptors located on vascular endothelial cells to stimulate excessive angiogenesis, thus allowing exponential tumor growth and providing a route for metastatic spread [25, 26]. By binding to VEGF, bevacizumab prevents VEGF from interacting with its receptors, and thus should inhibit new vessel growth. In preclinical models, bevacizumab blocked VEGF-induced cell proliferation, survival, and migration, reversed VEGF-induced vascular permeability, and normalized VEGF-induced changes in vessel architecture [27, 28]. These changes led to a reduction in interstitial pressure and increased blood flow, which may be important in improving the delivery of cytotoxic drugs used in combination with bevacizumab, and in reversing tumor hypoxia and its impact in mediating drug resistance. Interestingly, this effect may be short-lived, because tumor blood vessels eventually collapse after prolonged VEGF blockade, possibly leading to development of VEGF resistance [29].

Bevacizumab has demonstrated efficacy in metastatic colorectal cancer (mCRC), improving survival when added to a variety of cytotoxic platforms [30–32], and also in metastatic breast cancer [improving progression-free survival (10.97 mos. vs 6.11 mos.: HR = 0.498, $p < 0.001$) and possibly overall survival (HR = 0.674, $p = 0.01$) in combination with paclitaxel, but not capecitabine] [33, 34] and advanced non-small cell lung cancer (improving survival when added to carboplatin plus paclitaxel) [35].

Rituximab

Rituximab is a chimeric IgG_1 mAb directed against CD20, an antigen expressed on most B cells, including follicular B-cell lymphomas. In preclinical studies, binding of rituximab to CD20 has been associated with cell cycle regulation, altered expression of other cell surface molecules, and induction of apoptosis [36]. Although CD20 has been suggested to be a calcium channel involved in B-cell growth, its actual function is not entirely clear [37]. Therefore, it is not known whether any of the observed preclinical effects actually contribute to the clinical efficacy of rituximab [36].

Rituximab is effective in the treatment of lymphoma, providing progression-free and overall survival advantages when added to front-line cytotoxic chemotherapy for diffuse and other aggressive B-cell lymphomas [38, 39], as well as indolent forms of the disease (i.e., follicular) [40]. Rituximab is also an effective maintenance therapy in indolent lymphomas after response to initial therapy [41]. In addition, rituximab has produced promising results in the treatment of autoimmune diseases, including rheumatoid arthritis and certain lupus variants [42–44].

Ibritumomab tiuxetan and tositumomab also target CD20, but their efficacy may be related not only to target binding in itself, but also to their conjugated radioisotopes (see below).

Epidermal Growth Factor Receptor–Targeted Antibodies: Cetuximab and Panitumumab

The other available mAbs target members of the erbB family of growth factor receptors: cetuximab (Erbitux®, ImClone Systems Inc., Branchburg, NJ) and panitumumab (Vectibix™, Amgen Inc., Thousand Oaks, CA) are directed against the epidermal growth factor receptor (EGFR) and trastuzumab against human EGFR type 2 (HER-2). Following ligand binding, these receptors dimerize, leading to autophosphorylation, tyrosine kinase activation, and downstream signaling that ultimately leads to cell proliferation and tumor growth [45]. Cetuximab is a chimeric IgG_1 mAb; panitumumab is a human IgG_2 mAb. Both bind to the extracellular domain of EGFR, thereby acting as competitive antagonists of the natural ligands, EGF and transforming growth factor α [45, 46]. As a result, these mAbs block EGFR-mediated signaling, leading to G_1 cell cycle arrest as a result of hypophosphorylation of the retinoblastoma protein [47]. In addition, these mAbs induce downregulation of EGFR expression on the cell surface [45].

Panitumumab is active as a single agent in the treatment of mCRC multirefractory to cytotoxics [48], but its efficacy as part of combination regimens is less clear [49]. Cetuximab is effective, in combination with cytotoxics and as single agent, in mCRC refractory to one or more therapies [50–52]. Cetuximab is also active in head and neck cancers, significantly prolonging survival when added to radiation therapy [53].

Trastuzumab

Trastuzumab is a humanized IgG_1 mAb that targets HER-2, which is overexpressed in some breast cancers. Binding of trastuzumab disrupts HER-2 signaling and blocks cell cycle progression in the G_1 phase, leading to inhibition of tumor growth [54]. The blockade of cell cycle progression by trastuzumab is correlated with the expression of $p27^{Kip1}$, an inhibitor of the cyclin E–CDK2 complex that controls progression through G_1 [55]. It remains unclear whether trastuzumab promotes HER-2 internalization or downregulation.

Trastuzumab is effective against metastatic breast cancer tumors overexpressing the HER-2 target, and its addition to standard chemotherapy results in higher response rates and longer progression-free survival (PFS) and overall survival times in this patient subpopulation [56–58]. The role of trastuzumab may extend to maintenance therapy throughout different cytotoxic regimens, but the benefit of that strategy is still to be determined [59].

These target-specific mechanisms—inhibition of VEGF by bevacizumab, binding to CD20 by rituximab, blocking EGFR by cetuximab and panitumumab, and blocking HER-2 by trastuzumab—are mediated by the antigen-binding site of the mAb and may contribute to some or all of the observed clinical efficacy of these agents. However, clinical efficacy may also be a result of, at least in some part, antibody-specific mechanisms mediated through the Fc domain of the mAb.

Antibody-Specific Mechanisms

Immune-mediated effector mechanisms, specifically ADCC and CDC, may contribute to the clinical efficacy of certain mAbs. Although preclinical evidence points to the potential importance of these mechanisms, there are only limited data to show that these mechanisms may indeed be important in the clinical setting.

ADCC

The strongest evidence supporting ADCC as a clinically meaningful mechanism of certain therapeutic mAbs is based on studies evaluating the impact of different allelic variations of FcγRs (*FcγR* polymorphisms) on clinical response (Fig. 2) [60, 61]. Polymorphisms have been identified in several *FcγR* subclasses, including *FcγRIIIa* and *FcγRIIa* [37]. In *FcγRIIIa*, a point mutation at nucleotide

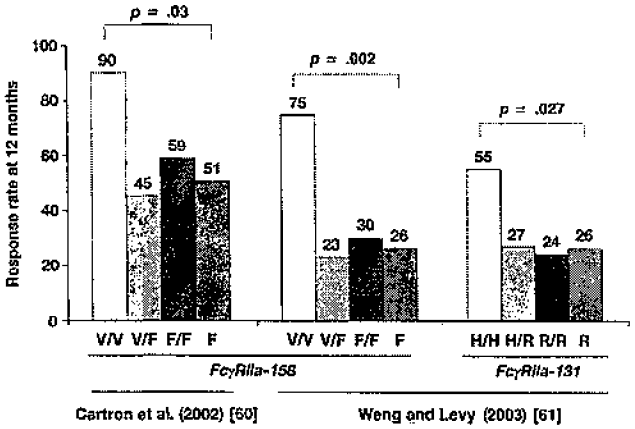

Figure 2. Response rates at 12 months with single-agent rituximab according to FcγR genotype in patients with follicular lymphoma. Cartron and colleagues [60] evaluated 49 patients receiving first-line rituximab. Weng and Levy [61] evaluated 87 patients, including 72 patients who had received chemotherapy prior to rituximab. Abbreviations: F, phenylalanine; H, histidine; R, arginine; V, valine.

559 results in substitution of valine by phenylalanine at amino acid 158. The FcγRIIIa-158V/V protein has higher affinity for IgG_1, IgG_3, and IgG_4 and shows greater NK cell–mediated activity than the FcγRIIIa-158F/F variant [62]. In *FcγRIIa*, which is expressed on macrophages but not NK cells, a point mutation changes arginine to histidine at position 131 and results in higher IgG binding affinity, particularly for IgG_2 [15]. On the basis of the different binding affinities, patients harboring FcγRIIIa-158V/V and FcγRIIa-131H/H would be expected to mount a more vigorous ADCC antitumor response upon mAb treatment.

Cartron and colleagues [60] tested *FcγRIIIa* polymorphisms in 49 patients with $CD20^+$ follicular non-Hodgkin's lymphoma who received first-line therapy with rituximab. Ten patients (20%) were homozygous for the *FcγRIIIa-158V/V* genotype and 17 patients (35%) for the *FcγRIIIa-158F/F* variant. The remaining 22 patients were heterozygous. Patients with the *FcγRIIIa-158V/V* genotype had significantly higher response rates than *FcγRIIIa-158F* carriers (i.e., homozygous *158F/F* or heterozygous *V/F*) when evaluated at 2 months (100% versus 67%; $p = .03$) or 12 months (90% versus 51%; $p = .03$). Moreover, the 3-year PFS rate tended to be higher in *FcγRIIIa-158V/V* patients than in *FcγRIIIa-158F* carriers, although the difference did not reach statistical significance (56% versus 35%; $p = .2$).

These findings were extended by Weng and Levy [61], who measured both *FcγRIIa* and *FcγRIIIa* polymorphisms in a cohort of 87 patients with follicular lymphoma, including 15 patients who received first-line rituximab and 72 patients previously treated with chemotherapy before rituximab. The cohort included 13 patients (15%) homozygous for *FcγRIIIa-158V/V* and 34 patients (39%) homozygous for *FcγRIIIa-158F/F*. Patients with the *FcγRIIIa-158V/V* genotype had a significantly higher response rate than *FcγRIIIa-158F* carriers when assessed during the first 3 months or at 6, 9, or 12 months (75% versus 26% at 12 months; $p = .002$). The 2-year PFS rate also favored the *FcγRIIIa-158V/V* homozygotes over *FcγRIIIa-158F* carriers (45% versus 12%; $p = .023$). When *FcγRIIa* polymorphism was evaluated, 20 patients (23%) were homozygous for *131H/H* and 24 patients (28%) for were homozygous for *131R/R*. Patients with the *FcγRIIa-116H/H* variant had a significantly higher 12-month response rate (55% versus 26%; $p = .027$) and 2-year PFS rate (37% versus 14%; $p = .011$) than *FcγRIIa-116R* carriers. On logistic regression, both *FcγRIIIa-158V/V* and *FcγRIIa-116H/H* polymorphisms were independently associated with a higher response rate and longer PFS time on rituximab. Taken together, these studies support a role for ADCC in the clinical efficacy of rituximab; they are also suggestive of a proof of principle in which ADCC may be particularly important in the efficacy of other IgG_1 mAbs, including cetuximab, as this isotype is the most potent ADCC mediator.

Recently, Zhang and colleagues [63] explored whether *FcγRIIa* and *FcγRIIIa* polymorphisms would influence clinical response to single-agent cetuximab in 39 patients with mCRC who had failed previous irinotecan- and oxaliplatin-based therapy. The cohort included five patients (13%) with the *FcγRIIIa-158V/V* genotype and nine patients (23%) with the *FcγRIIa-116H/H* variant. Only two patients (5%) had partial responses to cetuximab—consistent with response rates reported for patients with advanced CRC. Patients with the *FcγRIIIa-158 F/F* and *F/V* genotypes tended to have stable disease (57% and 71%, respectively), whereas those with the *158V/V* genotype tended to have progressive disease (80%) on treatment with cetuximab ($p = .082$). Similarly, patients with *FcγRIIa-116H/H* and *H/R* variants tended to have stable disease (78% and 71%, respectively), and those with *116R/R* tended to have progressive disease (86%) ($p = .082$). Similar patterns were seen for PFS and overall survival, although the predictive value of the polymorphisms considered independently did not reach statistical significance. When the polymorphisms were considered together, however, patients harboring the combination of *FcγRIIIa-158V/V* and *FcγRIIa-116R/R* had a significantly shorter PFS interval than the remaining patients (1.1 versus 3.7 months; $p = .004$).

In principle, the impact of *FcγR* polymorphisms on responsiveness to therapy supports a potential contribution of ADCC to antitumor efficacy for rituximab. Additional indirect evidence, albeit more limited, is beginning to indicate that ADCC may also be contributing to cetuximab activity.

Taken together, however, these studies suggest that specific polymorphisms may influence outcomes differently with cetuximab in mCRC than with rituximab in follicular lymphoma: the presence of the *FcγRIIa-116H* polymorphism seems to be indicative of a good response to therapy for both mAbs, while the *FcγRIIIa-158F* variant is linked to a poor response to rituximab in patients with hematologic tumors, but it seems to be linked to a potent response to cetuximab in patients with mCRC. Despite the small sample size in these studies, it is important to recognize that not all mAbs are the same, even those of the same isotype. Potential differences may relate to the actual determinants of the antibodies or the exact mechanism(s) of cytotoxicity that they evoke.

Clearly, we are at an early stage in our understanding of whether ADCC contributes to the clinical efficacy of mAbs, and if so, to which ones. The interplay between host and tumor is, in all likelihood, the major determining factor in any immune response directed against tumors. Recent reports indicate that CRC tumors with a high density of infiltrating immune cells are less likely to disseminate; furthermore, the type, density, and location of immune cells that infiltrate into colorectal tumors are robust predictors of clinical outcomes, and their prognostic value is independent from, and superior to, staging based on histopathological criteria [64]. These findings point to the importance of immune responses in controlling tumor growth, on which therapeutic antibodies could capitalize. It is also important to consider that the intrinsic susceptibility of various tumor cells (e.g., colon cancer cells versus lymphoma cells) to immune-mediated effector functions may differ, and it may change as disease progresses. It is tempting to speculate that the benefit of ADCC may be greatest at earlier stages of disease when the tumor burden is smallest. Accordingly, it may be unrealistic to expect a major contribution to antitumor activity from ADCC in studies conducted with single-agent monoclonal therapy in advanced cancer, where immune function may be impaired by previous treatments or the nature of the disease itself [65]. Additional studies at earlier stages of disease are needed.

In theory, ADCC is less likely to be involved in the clinical response to an IgG_2 mAb such as panitumumab, than to IgG_1 mAbs. The *FcγRIIa-131H* allele encodes the only receptor capable of efficiently interacting with IgG_2, and it is expressed on macrophages but not NK cells. Importantly, the FcγRIIIa receptor on NK cells, regardless of the *158V/F* polymorphism, binds poorly to IgG_2 [15]. Studies evaluating clinical response or outcome with panitumumab according to *FcγR* polymorphism have not been reported to date.

CDC

Evidence supporting a role of CDC in the clinical efficacy of therapeutic antibodies, specifically rituximab, is based largely on preclinical models. Rituximab cured all wild-type mice injected with human CD20-transfected murine EL4 thymoma cells, but its protective effects were abolished in C1q-deficient mice lacking an intact complement pathway [17]. In contrast, depletion of NK cells or neutrophils did not influence the protective effects of rituximab, nor did testing in athymic nude mice. Similar findings were recently reported in a murine B-cell lymphoma model using human CD20-transfected 38C13 lymphoma cells [66]. Rituximab cured all animals with no evidence of lymphoma when assessed by immunohistochemistry and polymerase chain reaction analysis, whereas its protective effect was abolished after complement depletion with cobra venom factor. Again, depletion of NK cells or neutrophils, or removal of phagocytic macrophages, did not affect the protective action of rituximab. These models strongly suggest that the protective effects of rituximab depend on CDC, at least in the mouse (as discussed before, immunological differences between murine and human systems preclude immediate extrapolation of conclusions from one to the other). However, the role of CDC in the clinical efficacy of rituximab or other therapeutic mAbs remains unclear [18, 66]. Studies evaluating the importance of CDC with other therapeutic mAbs have not been reported.

As noted previously, CDC is regulated by a series of membrane proteins that promote inactivation of C3b or prevent MAC formation [2]. The role of these inhibitors in regulating the clinical activity of rituximab was explored in 29 patients with follicular lymphoma, most of whom had been treated with two or three courses of chemotherapy before receiving rituximab. Overall, eight patients had complete responses, 11 patients had partial responses, and the remaining 10 patients had no or minimal responses. The expression of the complement inhibitors CD46, CD55, and CD59, whether assessed alone or in various combinations, did not differ across the three response groups. Moreover, rituximab-induced CDC did not differ across the three groups when assessed in vitro [67]. Thus, the role of CDC in the clinical activity of rituximab, if any, is unclear based on available data.

Future Trends: Optimizing Therapeutic Activity

Antibody Conjugates

Efforts to improve the cytotoxic actions of mAbs and consequently their therapeutic effectiveness have focused on conjugates with highly toxic substances, including radio-

isotopes and cytotoxic agents [6, 68, 69]. These conjugates can deliver a toxic load selectively to the tumor site while normal tissues are generally spared. In order to minimize toxicity, conjugates are usually engineered based on molecules with a short serum half-life (thus, the use of murine sequences, and IgG_3 or IgG_4 isotypes).

Two radioimmunoconjugates, ibritumomab tiuxetan (Zevalin®; Biogen Idec Inc., Cambridge, MA) and tositumomab (Bexxar®; GlaxoSmithKline, Research Triangle Park, NC) were approved in 2002 and 2003, respectively, in the U.S. for use in relapsed or refractory non-Hodgkin's lymphoma [70]. Both agents are murine mAbs that target the CD20 antigen on B-cell lymphoma cells (the same antigenic target as rituximab). Ibritumomab tiuxetan is an IgG_1 antibody that is conjugated to ^{90}Y, whereas tositumomab is an IgG_2 antibody containing ^{131}I [71].

The conjugation of a cytotoxic agent to a mAb is illustrated by gemtuzumab ozogamicin (Mylotarg®; Wyeth Pharmaceuticals Inc., Philadelphia, PA) which was approved in the U.S. in 2000 for treatment of acute myelogenous leukemia (AML). This humanized IgG_4 mAb targets the CD33 antigen expressed in AML blast cells and contains a calicheamicin γI derivative attached via a bifunctional linker [72]. Other conjugates of mAbs with cytotoxic toxins are under clinical evaluation [73]. For example, BL22 is a conjugate of an anti-CD22 mAb fragment and *Pseudomonas* exotoxin A, which has shown promising activity in chemoresistant hairy-cell leukemia [74]. Ricin has also been successfully conjugated with IgG_1 backbones targeted against CD22 (antibody RFB4–deglycosylated ricin A chain [dgA]) [75] and against CD19 (antibody HD37-dgA) [76]. Both of these molecules have clinical activity, and a combination is now under development for the treatment of pediatric and adult acute lymphoblastic leukemia [77].

Thus, mAb conjugates are a viable approach to killing tumor cells in hematological malignancies. However, this approach may be more problematic in solid tumors, where it may be difficult to deliver a sufficient amount of cytotoxic agent to achieve meaningful tumor regression [68]. Interestingly, the effect of conjugating a toxin or a radioisotope on the effector functions (ADCC or CDC) of an Ig moiety has not been consistently evaluated.

Enhancing Antibody Structure

Several strategies have been used to alter antibody structure in order to increase immune-mediated effector functions. The Fc region of IgG antibodies contains oligosaccharides that influence the orientation of the heavy chains, prevent interactions between adjacent domains, and allow exposure of key sugar residues on the antibody surface [78]. By maintaining the Fc domain in an open configuration, the presence of oligosaccharides—specifically, the N-linked oligosaccharide at asparigine-297 in the C_H2 domain of IgG_1—is important for binding to FcγR as well as C1q [2, 4, 79]. Recombinant mAbs are commonly produced in Chinese hamster ovary cell lines, which generate oligosaccharides having a high fucose content [2, 80]. Through glycoengineering, mAbs with low fucose content can be produced. A defucosylated IgG_1 mAb against the chemokine receptor 4 exhibited greater binding to FcγRIIIa, greater ADCC using human peripheral blood mononuclear cells or NK cells as effectors, greater phagocytic activity by monocytes and macrophages, and greater antitumor activity in murine models of T-cell leukemia and lymphoma than highly fucosylated IgG_1 mAbs [80, 81].

Modification of amino acids within the C_H2 domain of the Fc region is another strategy for enhancing immune-mediated effector functions. On high resolution mapping, several amino acids, all of which are located in the C_H2 domain near the hinge region, were identified as being important in IgG_1 binding to FcγR [82, 83]. Several additional amino acids were also important in IgG_1 binding to FcγRII and FcγRIII. By changing these amino acids to alanine, variants with altered binding characteristics to FcγRII and FcγRIII were identified. The binding of IgG_1 to FcγRIIIa, the main receptor mediating ADCC by NK cells, was 51% greater when simultaneous alanine mutations were made at Ser298, Glu333, and Lys334. Notably, this mutant exhibited greater ADCC mediated by NK cells, with cytotoxicity comparable to a 10-fold higher concentration of native IgG_1 [82]. It is hoped that by combining these antibody engineering strategies—reduced fucosylation and amino acid substitutions—it may be possible to generate mAbs with superior FcγR binding characteristics, leading to more effective ADCC, which will ultimately translate into higher response rates and more durable responses, particularly in patients with solid tumors.

Conclusion

mAbs represent an important advance in the treatment of certain hematologic malignancies and solid tumors. Unlike many small molecules, mAbs offer unique target specificity. The field has evolved rapidly in recent years, and now it is much easier to create mAbs against a variety of targets of potential relevance to tumor growth and survival. Targets, including CD20, HER-2, VEGF, and EGFR, have now been validated by the clinical efficacy of mAbs. In the future, the list of viable targets is likely to expand. Two areas that may merit more attention are membrane transporters and stromal function. Positron emission tomography scans detecting selective uptake of fluoroglucose by tumor cells are

already being used diagnostically. By targeting specific transporters, mAbs may eliminate the ability of tumor cells to survive in a nutrient-challenged environment. The stroma is the interface between tumor and host, and accordingly, mAbs against stromal antigens may make it more resistant to the onslaught of tumor cells.

At first glance, the clinical efficacy of mAbs may be attributed to target-specific effects. By binding to their target, mAbs neutralize an important factor or receptor that drives cell proliferation and tumor growth. However, the therapeutic activity of mAbs may go beyond these target-related effects. Currently available mAbs are IgG antibodies, and consequently, they have the potential to activate immune-mediated effector functions, including ADCC and CDC. ADCC occurs when target-bound antibodies mobilize effector cells via interaction of their Fc domain with FcRs on the surface of immune cells. The interaction between the Ig Fc domain and FcRs on immune cells depends on the Fc domain (its sequence and glycosylation) and on the FcR structure (types and polymorphisms). Thus, the binding affinity of IgG for the FcgR mediating ADCC and other effector mechanisms varies by antibody isotype, and antibody-related effects may not be equal for all IgG isotypes or for all mAbs within a given isotype. The highest binding affinity for the various FcgR subclasses is found with IgG_1 and IgG_3, and therefore mAbs of these isotypes should be most likely to stimulate immune-mediated effector functions. Also, the intensity of ADCC is expected to fluctuate depending on the allelic *FcR* variants present in the host, and preliminary evidence points to an effect of those polymorphisms in clinical response to mAbs. Finally, by modifying antibody glycosylation patterns or amino acid sequence in the Fc domain, it may be possible to further enhance antibody-related effects, and hopefully, improve clinical efficacy of future mAbs.

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Targeted Therapy of Cancer: New Prospects for Antibodies and Immunoconjugates

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EXHIBIT 2

Targeted Therapy of Cancer: New Prospects for Antibodies and Immunoconjugates[1]

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ABSTRACT Immunotherapy of cancer has been explored for over a century, but it is only in the last decade that various antibody-based products have been introduced into the management of patients with diverse cancers. At present, this is one of the most active areas of clinical research, with eight therapeutic products already approved in oncology. Antibodies against tumor-associated markers have been a part of medical practice in immunohistology and in vitro immunoassays for several decades, have even been used as radioconjugates in diagnostic imaging, and are now becoming increasingly recognized as important biological agents for the detection and treatment of cancer. Molecular engineering has improved the prospects for such antibody-based therapeutics, resulting in different constructs and humanized/human antibodies that can be administered frequently. Consequently, a renewed interest in the development of antibodies conjugated with radionuclides, drugs, and toxins has emerged. We review how antibodies and immunoconjugates have influenced cancer detection and therapy, and also describe promising new developments and challenges for broader applications. (*CA Cancer J Clin 2006;56:226–243.*) © *American Cancer Society, Inc., 2006.*

INTRODUCTION

The search for a mechanism to target diseases selectively was first realized when resistance to infectious disease could be transferred from one animal to another through their serum, a process known as passive serotherapy.[1] Five years later, in 1895, Hericourt and Richet immunized dogs with a human sarcoma and then transferred the serum to patients.[2] This anticipated the "magic bullet" concept of Paul Ehrlich in 1908, that "toxins" could be targeted to cancer and other diseases.[3] Another half-century passed before antibodies were identified as the substance in serum responsible for these effects.

Despite being potent immune system instigators for killing infectious agents, clinical research initially focused on immunoconjugates prepared with radionuclides, drugs, or toxins, since unconjugated or "naked" antibodies had little therapeutic benefit in oncology compared with the immunoconjugates. Early immunotherapy trials failed to show substantial responses,[4–6] but antibodies against carcinoembryonic antigen (CEA) could selectively target and disclose sites of CEA-expressing cancers in patients, and also deliver cytotoxic radioactivity in human colonic cancer xenografts having CEA.[7,8] Thereafter, DeNardo, et al.[9] reported responses in lymphoma patients to radiolabeled antibodies, and soon others confirmed that radiolabeled antibodies had antitumor activity in non-Hodgkin lymphoma (NHL), but there was also early evidence that the naked antibodies themselves might be effective.[10–12] It was during this same period that rituximab (Rituxan, Genentech, and biogen idec), an anti-CD20 IgG, became of interest as a therapeutic for NHL without being radiolabeled.[13] The experience and subsequent introduction of rituximab into the treatment of NHL can be credited for the expanded interest in unconjugated antibodies for cancer therapy.

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Antibodies (eg, IgG, which is the most commonly used immunoglobulin form, Figure 1) are unique proteins with dual functionality. All naturally occurring antibodies are multivalent, with IgG having two binding 'arms.' Antigen-binding specificity is encoded by three *complementarity-determining regions* (CDRs), while the *Fc-region* is responsible for binding to serum proteins (eg, complement) or cells. An antibody itself usually is not responsible for killing target cells, but instead marks the cells that other components or effector cells of the body's immune system should attack, or it can initiate signaling mechanisms in the targeted cell that leads to the cell's self-destruction (Figure 2). The former two attack mechanisms are referred to as antibody-dependent *complement-mediated cytotoxicity* (CMC) and *antibody-dependent cellular cytotoxicity* (ADCC). ADCC involves the recognition of the antibody by immune cells that engage the antibody-marked cells and either through their direct action, or through the recruitment of other cell types, lead to the tagged-cell's death. CMC is a process where a cascade of different complement proteins become activated, usually when several IgGs are in close proximity to each other, either with one direct outcome being cell lysis, or one indirect outcome being attracting other immune cells to this location for effector cell function.

Antibodies, when bound to key substances found on the cell surface, also can induce cells to undergo *programmed cell death*, or *apoptosis* (Figure 2). For example, if rituximab binds to two CD20 molecules, this triggers signals inside the cell that can induce apoptosis.[14] If rituximab is cross-linked by other antiantibodies, the apoptotic signal is intensified.[15] This cross-linking could also occur when the antibody is bound by another immune cell through its Fc-gamma receptors (FcγR). Other antibodies, such as trastuzumab (anti-HER2/*neu*; Herceptin, Genentech) and cetuximab (anti-epidermal growth factor receptor, EGFR; Erbitux, ImClone Systems and Bristol-Myers Squibb) also have the ability to inhibit cell proliferation.[16–18] Because cells frequently have alternative pathways for critical functions, interrupting a single signaling pathway alone might not be sufficient to ensure cell death. From this perspective, it is not surprising that antibodies are often best used in combination with chemotherapy and radiation therapy to augment their antitumor effects.[19–21]

Bevacizumab (Avastin, Genentech) is yet another example of how antibodies can be used therapeutically. This antibody binds to vascular endothelial growth factor (VEGF) that is made by tumor cells to promote vessel formation, thereby preventing it from interacting with endothelial cells to form new blood vessels (Figure 2).[22] Antibodies can also be used to modulate immune response. Antibodies to the cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) stimulate T-cell immune responses by blocking the inhibitory effects of CTLA-4, which can enhance tumor rejection.[23] However, release of this innate inhibitory mechanism can also increase the risk of autoimmunity.[24] Two human anti-CTLA-4 antibodies are currently in early clinical trials (MDX-010, Medarex, and CP-675,206, Pfizer), with evidence that they may have activity in melanoma.[24] There are already a number of antibodies used or being studied as therapeutic agents in cancer as well as autoimmune diseases (eg, alemtuzumab, daclizumab, infliximab, rituximab, epratuzumab).[25–31] Antibodies also can block molecules associated with cell adhesion, thereby inhibiting tumor metastasis.[32,33] With such diverse mechanisms of action, there are a number of opportunities for building antibody-based therapeutics.

Antibodies naturally have long serum half-lives. For immunotherapy, this property is helpful because the antibody is maintained in the body fluids, where it can continually interact with its target. For other targeting strategies, most notably with radioconjugates, it can be harmful because the highly radiosensitive bone marrow is continually exposed to radiation, resulting in dose-limiting myelosuppression. The large size of an antibody impacts its ability to move through a tumor mass. A high interstitial pressure inhibits the diffusion of larger molecules within the tumor.[34] Migration within the tumor is also inhibited by a binding-site barrier, a process where the antibody as it is leaving the tumor's blood vessels binds to the

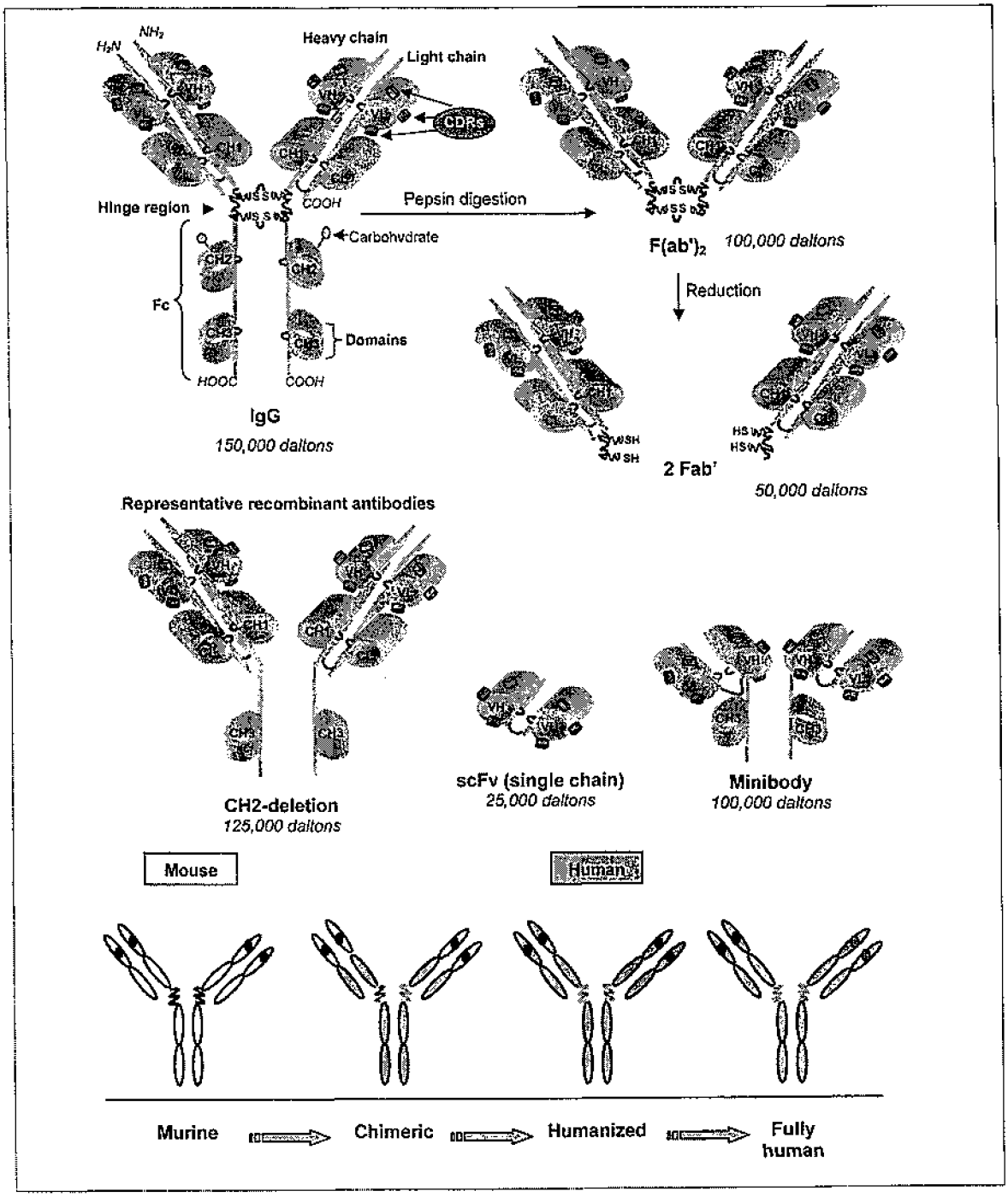

FIGURE 1 Schematic representation of an IgG molecule, its chemically produced fragments, and several recombinant antibody fragments with their nominal molecular weights. At the bottom, a schematic representation of the process involved in engineering murine MAbs to reduce their immunogenicity is provided. A chimeric antibody splices the VL and VH portions of the murine IgG to a human IgG. A humanized antibody splices only the CDR portions from the murine MAb, along with some of the adjacent "framework" regions to help maintain the conformational structure of the CDRs. A fully human IgG can be isolated from specialized transgenic mice bred to produce human IgG after immunizing with tumor antigen or by a specialized phage display method.

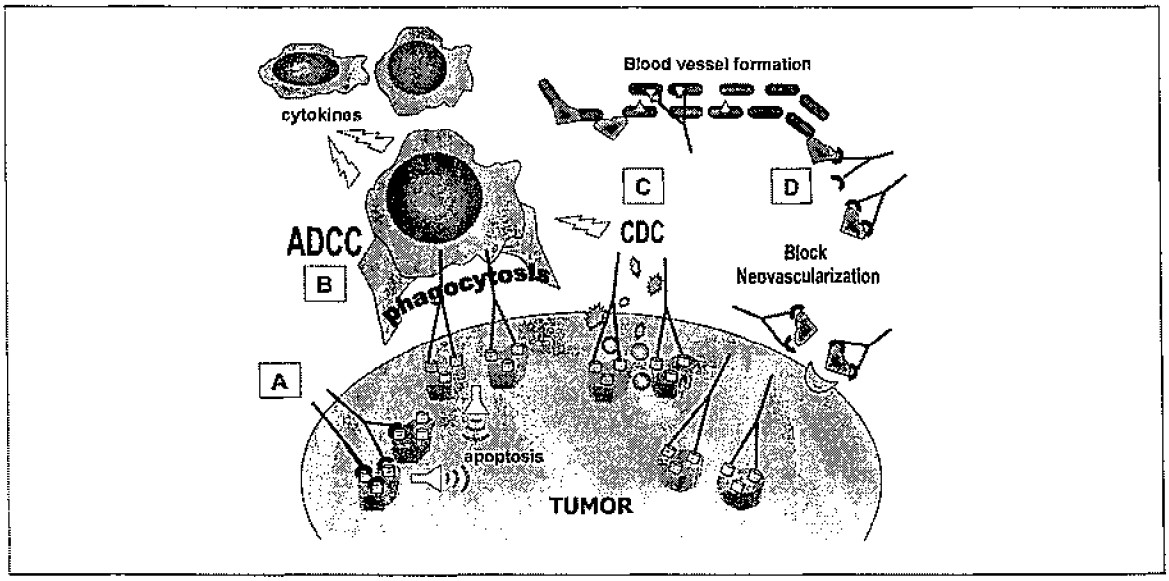

FIGURE 2 Mechanisms of action associated with unconjugated antibodies. In this example, the antigen is shown to be floating in lipid rafts within the tumor cell membrane. (A) Antibodies can activate apoptotic signals by cross-linking antigen, particularly across different lipid rafts. Additional cross-linking of antibody by immune cells can also enhance cellular signaling. (B) Immune cells themselves can attack the antibody-coated cell (eg, phagocytosis), and/or they can liberate additional factors, such as cytokines that attract other cytotoxic cells. (C) If antibodies are positioned closely together, they can initiate the complement cascade that can disrupt the membrane, but some of the complement components also are chemo-attractants for immune effector cells and stimulate blood flow. (D) Tumors also can produce angiogenic factors that initiate neovascularization. Antibodies can neutralize these substances by binding to them, or they can bind directly to unique antigens presented in the new blood vessels, where they could exert similar activities.

first available antigen, concentrating the antibody in the perivascular space.[35] High-affinity antibodies are less likely to migrate into the tumor bed.[36] Administering higher doses of the antibody can reduce the effect of the binding site barrier and allow the antibody to diffuse more deeply into the tumor bed.[37] For cytotoxic agents that must be internalized to kill the cell (eg, toxins, cytotoxic drugs), the ability to distribute throughout the tumor is important. Radioconjugates are less affected by this because some radioactive particles can traverse as much as 1.0 cm from where they are deposited (bystander or crossfire effect).

THERAPY WITH UNCONJUGATED ANTIBODIES

A renewed interest in the effects of unconjugated antibodies in cancer began in the early 1980s, after murine monoclonal antibodies (MAbs) became available.[38] These initial trials were performed in hematological malignancies, as well as in colorectal cancer and melanoma.[4–6,39–41] As with many innovative treatment approaches that are sometimes introduced before the technology has matured sufficiently to extract maximum benefit, only occasional clinical responses were observed. With insufficient efficacy and the immunogenicity of the foreign murine MAb, most of these studies were terminated. Fortunately, some investigators persevered. An excellent lesson on the tribulations of the development of an antibody product between an academic group and industry is that of alemtuzumab (Campath, Berlex, and Genzyme).[42] Alemtuzumab (anti-CD52) had one of the earliest and protracted developments of an antibody ultimately commercialized. It took over 20 years from the development of the first rat immunoglobulin against CD52, changing the immunoglobulin type, and finally developing a humanized, recombinant form, and involved several commercial firms during this time. Chemotherapy-refractive chronic lymphocytic leukemia was the indication finally approved in 2001.

Due in part to the contributions made by the groups led by Morrison (Columbia and Stan-

ford Universities) and Winter (Cambridge). MAbs now are engineered to remove a significant portion of the murine component of the IgG, substituting human IgG components before entering clinical studies.[43–45] Chimeric antibodies essentially splice V_L and V_H regions on the murine antibody to the human IgG, making a molecule that is 75% human and 25% murine IgG, whereas a humanized antibody grafts the CDR regions from a murine MAb, along with some of the surrounding "framework" regions to maintain CDR conformation, onto a human IgG, essentially making a molecule with 5% of its sequence from the parental MAb (Figure 1).[15] More recent advances have made available, either by genetic or phage-display methods, the development of fully human MAbs that have now entered clinical trials.[46] Such engineered MAbs are postulated to greatly reduce the immunogenicity of antibodies, allowing multiple injections to be given, and the human Fc enhances the interaction with other immune system elements.

Rituximab is perhaps the most prominent example of a highly successful paradigm of antibody therapy. As a chimeric antibody, not only did it have reduced immunogenicity, but its effector function (associated with the Fc-portion) was improved. For example, when testing ADCC activity against follicular lymphoma isolated from 43 patients, Weng, et al. reported that only rituximab, not its parent murine anti-CD20 IgG (2B8), had activity in vitro.[47] Rituximab was initially approved as a single agent therapy for relapsed or refractory low-grade, follicular B-cell NHL, having an overall response rate of 48% (10% were complete responses, CR) with a median duration of 11.8 months.[48,49] Since CD20 is not expressed on precursors B-cells, rituximab induces a depletion of only mature B-cells. Rituximab's major side effects, which are thought to be associated with the activation of complement pathways, occur during or shortly after its infusion. Other less common side effects include symptoms associated with tumor lysis syndrome, severe mucotaneous reactions, renal toxicity, cardiac arrhythmias, hypersensitivity reactions, and reactivation of hepatitis B (primarily when used in combination with chemotherapy).[49]

Rituximab's activity is unique among cancer treatments because 40% of the patients retreated with rituximab could again respond with a similar duration.[50] Extending the duration of rituximab therapy can improve the response rate, particularly the number of complete responses, and its duration. However, whether given as a maintenance regimen or retreating at the first sign of progression, the time to chemotherapy was the same.[51] With both approaches having equal benefit, retreatment is generally favored because of the higher expense of a maintenance regimen. Despite the success of rituximab as a monotherapy, there are still a number of patients who do not respond to the initial treatment, and over time, many of those who do will relapse. In an attempt to improve outcome, rituximab has been combined with chemotherapy regimens, including CHOP, CVP, and MCP, as front-line treatments, with very promising results in not only follicular B-cell lymphomas, but also in diffuse large B-cell lymphomas.[52,53] Indeed, trials examining front-line combinations of rituximab and chemotherapy have already demonstrated improvements in response rates, time to progression, and event-free survival, and while the overall response rates are promising based on current 2- to 3-year follow-up data, more time will be required to fully appreciate its impact.[52] Even in chronic lymphocytic leukemia (CLL), where initial testing of rituximab was disappointing, dose intensification and combinations with chemotherapy have provided significant improvements in response.[54,55] Early clinical studies combining rituximab with a humanized anti-CD22, epratuzumab (Immunomedics, Inc.) suggested the potential for additional benefit, particularly in patients with diffuse large B-cell lymphomas.[56,57] Studies have also assessed the possible role of an anti-CD80 MAb (galiximab, biogen idec) as a monotherapy in NHL,[58,59] and clinical trials are in progress testing its combination with rituximab.

Considerable attention has been devoted to understanding the mechanism of action of rituximab, particularly why some B-cell lympho-

mas are affected and why not all patients with follicular lymphomas respond. As mentioned earlier, rituximab has been shown to have CMC, ADCC, and apoptotic activity, with the former two mechanisms believed to have the greatest impact, although there are conflicting views of which of these two pathways contributes the most to the response.[14,60–66] Studies in transgenic and other mouse models have supported the importance of the Fc-receptor-mediated mechanism of action for rituximab.[67,68] These efforts have contributed in part to a better understanding of the role of various Fc receptors found on a variety of immune effector cells (eg, B-cells, neutrophils, natural killer cells, and monocytes) on (in the case of rituximab) the clearance of B-cells, as well as the plasma half-life of antibodies.[69] Not only do the various Fc-receptors influence binding, but the absence of certain carbohydrates on the Fc portion of the IgG can affect both ADCC and CMC activities.[70,71] Cartron, et al. found that the expression of the homozygous Fc-gamma RIIIa receptor (CD16) 158V genotype correlated with a higher response rate to rituximab, but it did not have an impact on the progression-free survival.[72] Weng, et al. found a similar correlation and also noted that the homozygous expression of the Fc-gamma RIIa histidine/histidine genotype correlated *independently* with a *higher* response rate, particularly when assessing the response status ≥6 months from treatment.[17] By unraveling the molecular basis for antibody cytotoxicity, not only can more effective antibodies be designed, but it could lead to a more rational approach for combinations to enhance activity, such as the finding that G-CSF up-regulates CD64 (Fc-gamma receptor I), which can enhance the binding of neutrophils and monocytes to B-cells coated with rituximab.[73] IL-12 has a similar stimulatory effect in mouse models and more recently has been applied clinically with promising results.[74,75] These discoveries are also having an impact on the development of antibodies for treating other cancers.[76–80]

The approved antibodies listed in Table 1 indicate that immunotherapy is not restricted to hematological malignancies, but includes diverse target antigens and receptors having different biological functions. Trastuzumab is an anti-HER2/*neu* antibody that has had a major impact on the therapy of breast cancer and is used alone and in combination with drugs.[81–83] HER2/*neu* is overexpressed on a proportion of breast and other cancers, and trastuzumab binds with an extracellular epitope of this target molecule. About 15% of women whose tumors overexpress HER2/*neu* respond to trastuzumab, but its efficacy is clearly best when used in combination with chemotherapy, where a 25% increase in the median survival (to 29 months) has been reported.[81] Further, the addition of this antibody to adjuvant chemotherapy for breast cancer has improved survival markedly.[83] Since only a portion of breast cancer patients overexpress HER2/*neu* and respond to trastuzumab, selection of suitable patients is important. New data are emerging that suggest trastuzumab treatment after adjuvant chemotherapy can have a significant benefit compared with observation, particularly in reducing the rate of distant recurrence.[82]

As a member of a family of receptor tyrosine kinases, the binding of HER2 by trastuzumab can interrupt intracellular signaling and affect tumor cell growth. Izumi, et al. showed that trastuzumab also has antiangiogenic properties.[84] While this may be an important underlying mechanism of action, other evidence suggests that trastuzumab's activity is principally governed by ADCC.[85] However, trastuzumab combined with chemotherapy improves response rates, despite the immunosuppressive activity of the chemotherapy, and trastuzumab's activity is enhanced when combined with other, nonantibody, Erb inhibitors, such as gefitinib and erlotinib, all of which suggest that its ability to interfere with signaling is important.[86] Since HER2 is a member of a family of growth factors known as the neuregulins/heregulin and is expressed in multiple neuronal and non-neuronal tissues in embryos and adult animals, including the heart, it is not surprising that cardiomyopathy has been associated with trastuzumab, particularly when combined with paclitaxel and anthracyclines.[87–90]

EGFR is also overexpressed in many solid cancers, and when bound by its ligand, cell growth is stimulated. However, when engaged by an EGFR-specific antibody, receptor phos-

TABLE 1 FDA-approved Antibodies for the Parenteral Use in Detection and Treatment of Cancer

Generic Name	Trade name	Agent/Target	Cancer Indication	Approval
Unconjugated				
Rituximab	Rituxan	Chimeric anti-CD20 IgG_1	B-cell lymphoma	1997
Trastuzumab	Herceptin	Humanized anti-HER2 IgG_1	Breast	1998
Alemtuzumab	Campath-1H	Humanized anti-CD52	CLL†	2001
Cetuximab	Erbitux	Chimeric anti-EGFR	Colorectal	2004
			Head/neck	2006
Bevacizumab	Avastin	Chimeric anti-VEGF	Colorectal	2004
Radioconjugates				
Satumomab pendetide	OncoScint*	^{111}In-murine anti-TAG-72 IgG	Colorectal, ovarian	1992
Nofetumomab merpentan	Verluma*	^{99m}Tc-murine anti-EGP-1 Fab'	SCLC‡	1996
Arcitumomab	CEA-Scan*	^{99m}Tc-murine anti-CEA Fab'	Colorectal	1996
Capromab pendetide	ProstaScint	^{111}In-murine anti-PSMA	Prostate	1996
Ibritumomab tiuxetan	Zevalin	^{90}Y-murine anti-CD20 IgG + rituximab	B-cell lymphoma	2002
Tositumomab	Bexxar	^{131}I-murine anti-CD20 IgG + unlabeled tositumomab	B-cell lymphoma	2003
Drug conjugates				
Gemtuzumab ozogamicin	Mylotarg	Humanized anti-CD33 IgG_4 conjugated to colicheamicin	AML§	2000

*No longer commercially available.
†CLL = chronic lymphocytic leukemia.
‡SCLC = small cell lung cancer.
§AML = acute myelogenous leukemia.

phorylation is decreased and cell growth is inhibited. The chimeric antibody against EGFR, cetuximab, also has an effect on neovascularization.[91,92] Cetuximab works best in combination with chemotherapy in colorectal cancer, for which it was initially approved, and with external irradiation in head and neck cancers, which was recently FDA-approved.[17,93] Beside the usual risks associated with antibody infusions, cetuximab causes an acneform rash and other skin reactions in most patients, with 10% of these being severe. There is evidence suggesting that the intensity of the skin rash is associated with its antitumor response and even survival.[94] Other EGFR antibodies, particularly humanized and fully human forms, also are in development, as indicated in Table 2, and may in fact be better tolerated and show evidence of activity without being combined with cytotoxic chemotherapy, which is currently being evaluated in Phase III trials. It is too early to speculate whether they will, in fact, provide any therapeutic advantages over cetuximab.

Bevacizumab targets and blocks vascular endothelial growth factor (VEGF) and VEGF's binding to its receptor on the vascular endothelium. Since VEGF is released by many cancers to stimulate proliferation of new blood vessels, the combination of bevacizumab and chemotherapy was found to increase objective responses, median time to progression, and survival in patients with metastatic colorectal cancer, compared with chemotherapy alone, but earlier preclinical studies indicated that anti-VEGF antibodies were active alone, as well as in combination with radiation.[22,95,96] It is currently being studied clinically in renal cell, breast, and lung cancers, as well as in a number of other nonhematological and hematological malignancies.[97–99] As might be expected, bevacizumab may cause gastrointestinal perforations and delayed wound healing, as well as hemorrhagic events (primarily seen in small cell lung cancer trials, where bevacizumab is not approved). Arterial thromboembolic events (eg, cerebral infarction, transient ischemic attacks, myocardial infarction, angina) and proteinurea also have been reported.[100]

IMMUNOCONJUGATES

Antibodies also function as carriers of cytotoxic substances, such as radioisotopes, drugs, and toxins (Figure 3). In NHL, anti-CD20 radioconjugates have superior antitumor activity compared with their unconjugated antibody counterparts, but there is increased, albeit manageable, hematological toxicity.[101,102] These findings are

TABLE 2 Selected Unconjugated Antibody Therapeutics in Advanced Clinical Testing

Generic Name	Agent/Target	Cancer
Apolizumab	Human anti-HLA-DR	CLL*, SLL**
Chimeric 14.18	Chimeric anti-ganglioside (GD2)	Neuroblastoma
Epratuzumab	Humanized anti-CD22	NHL§
Galiximab	Humanized anti-CD80	NHL§
HuMax-CD4	Fully human anti-CD4	CTCL‡
Lumiliximab	Humanized anti-CD23 (Fc-epsilon RII)	CLL*
MDX-010	Anti-CTLA-4	Melanoma
Matuzumab	Humanized anti-EGFR	CRC†
Orgegovomab	Murine anti-CA-125	Ovarian
Panitumumab	Human anti-EGFR	NSCLC¶, CRC†, renal
Pertuzumab	Humanized anti-HER-2	Breast, prostate, ovarian
Rencarex	Chimeric anti-G250	Kidney
Vitaxin	Humanized anti-$\alpha v\beta 3$ integrin	Melanoma, prostate

*CLL = chronic lymphocytic leukemia.
†CRC = colorectal cancer.
‡CTCL = cutaneous T-cell lymphoma.
§NHL = non-Hodgkin lymphoma.
¶NSCLC = non-small cell lung cancer.
**SLL = small lymphocytic lymphomas.

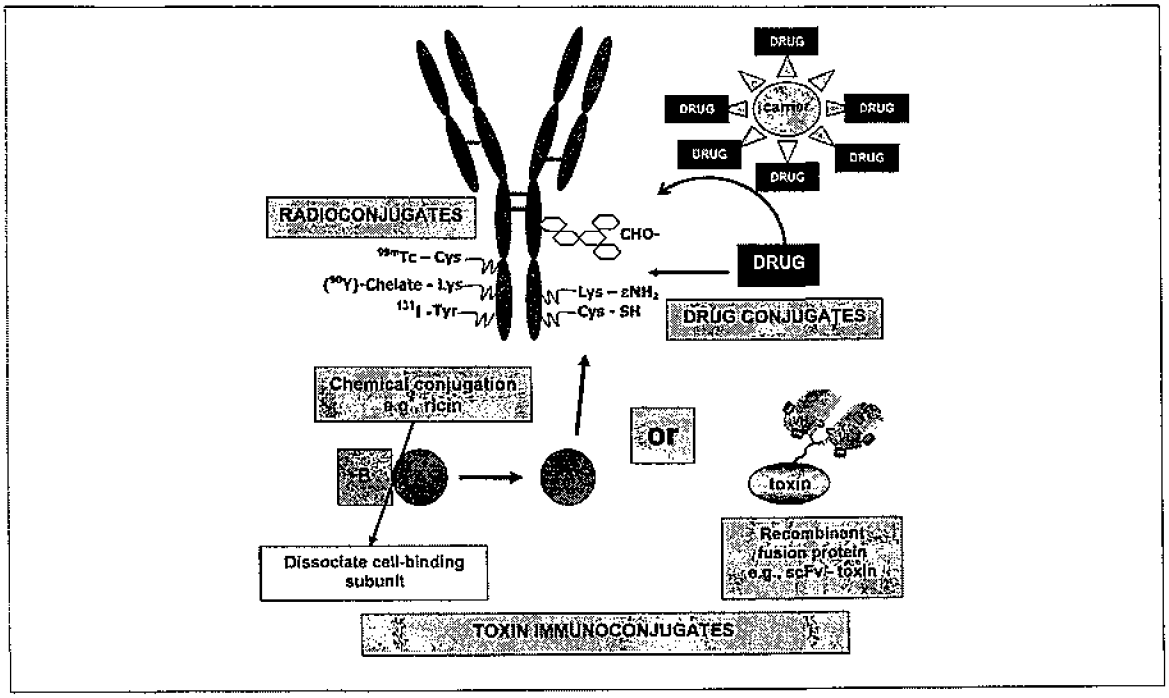

FIGURE 3 Immunoconjugates are formed primarily by chemical reactions. Radioconjugates can be formed by coupling radioiodine to tyrosine residues, or by binding chelates to lysine residues, which are then used to bind a variety of radiometals, such as ^{90}Y. Cysteine residues are also useful for coupling radionuclides, particularly ^{99m}Tc and rhenium, but cysteine is also used for conjugation of drugs and toxins, which can also be coupled to lysine residues. In addition, the carbohydrates found on IgG can be modified to allow the coupling of chelates or drugs. Drugs have also been coupled to an intermediate carrier that allows for a higher number of drugs to be bound to the antibody. Toxin conjugates usually need to be modified to remove their innate cell binding properties, with the biologically active portion then coupled to the antibody or used as a portion of a recombinant fusion protein.

strong incentives to continue the pursuit of immunoconjugates for cancer therapy.

Radionuclides

Radiolabeled antibodies were the first group of immunoconjugates to be examined.[103] Table 3 lists some of the more commonly used radionuclides conjugated to antibodies for cancer treatment. Because the radioactivity can be detected easily by external scintigraphy, it is also noteworthy to mention the additional application of radiolabeled antibodies for imaging. The demonstration of cancer targeting with a radiolabeled antibody fragment to CEA resulted in the development of radiolabeled antibodies for cancer imaging.[7] Since then, ^{99m}Tc- and ^{111}In-radioconjugates have been commonly used for this application, but with the advent of positron-emission tomography (PET), investigators are now beginning to take advantage of this technologically superior imaging system by radiolabeling tumor-associated antibodies with positron-emitters.[104–107]

Whereas external beam radiation delivers a focused beam of high dose rate radiation for short bursts that are divided over several weeks and is designed to treat local disease, *radioimmunotherapy* (RAIT) is typically given as an intravenous injection, thereby allowing radioactivity to be delivered to tumors throughout the body. Tumor uptake of a radiolabeled IgG occurs gradually, taking 1 to 2 days before peak uptake occurs. Peak uptake is typically <0.01% of the total injected dose per gram tumor, but the radioactivity deposited in the tumor can be detected several weeks later.[108] Because of its kinetics, the radiation-absorbed dose delivered by RAIT occurs at a much lower dose rate than external beam irradiation, but is continually present for a period of time defined by the physical half-life of the radionuclide and the biological half-life of the antibody residing in the tumor. This continuous, low dose rate radiation exposure can be as effective as intermittent, high dose rate radiation.[109,110]

When it comes to choices of radionuclides for therapy, tumor size is the primary consideration. Medium-energy beta-emitters, such as ^{131}I (0.5 MeV) and ^{177}Lu (0.8 MeV), can traverse 1.0 mm, while high-energy beta-emitters, such as ^{90}Y or ^{188}Re (2.1 MeV), can penetrate up to 11 mm, making it possible for beta-emitters to kill across several hundred cells, referred to earlier as a *bystander* or *crossfire* effect.[111] This is considered a significant attribute for radioconjugates compared with other immunoconjugates, since they can be therapeutically active even if heterogeneous antigen expression, tumor architecture, or other factors impede targeting of every cell. Although higher energy beta-emitters have the potential of killing cells across a longer path-length, the absorbed fraction is higher for the lower energy beta-emitters (ie, probability of hitting the nuclear DNA), making them efficient killers. Alpha-emitters, such as ^{213}Bi and ^{211}At, traverse only a few cell diameters, but an alpha particle is also a far more efficient (energetic) killer than even a low-energy beta particle, requiring fewer "hits" to damage cellular processes.[111] Low-energy electrons, such as are produced by Auger emitters (^{125}I, ^{67}Ga, or ^{111}In, for example) have to be in close contact, preferably inside a cell or in the nucleus, to exert a cytotoxic effect. As one might expect, beta-emitters are most likely best applied in situations where the tumors are ≥0.5 cm in diameter; otherwise a substantial portion of the energy from the radioactive decay will be absorbed in the surrounding normal tissue. The alpha and low-energy electron emitters are best applied when the disease burden is smaller, more localized, or where there may be single or small clusters of cells (eg, leukemia, malignant ascites).[112,113]

The primary concern for using radionuclide-labeled IgG is that it remains in the blood for an extended period of time, which continually exposes the highly sensitive red marrow to radiation, resulting in dose-limiting myelosuppression. Smaller forms of the antibodies, such as a $F(ab')_2$ or Fab', and more recently, molecularly engineered antibody subfragments (Figure 1) with more favorable pharmacokinetic properties, are removed more rapidly from the blood, thereby improving tumor/blood ratios.[114,115] There have been reports of improved therapeutic responses using smaller-sized antibodies, but these smaller entities frequently are cleared from the blood by renal filtration, and as a result, many radionuclides (eg, radiometals) become trapped in a higher concentration in the kidneys than in the

TABLE 3 Physical Properties of Several Examples of Radionuclides Used for Radioconjugate Therapy

Radionuclide	Emission	Half-life	Range[127]	Approximate # Cell Diameters*
^{131}Iodine	β	8.0 d	0.08–2.3 mm	10 to 230
^{90}Yttrium	β	64.1 h	4.0–11.3 mm	400 to 1100
^{177}Lutetium	β	6.7 d	0.04–1.8 mm	4 to 180
^{188}Rhenium	β	17.0 h	1.9–10.4 mm	200 to 1000
^{67}Copper	β	61.9 d	0.05–2.1 mm	5 to 210
^{211}Astatine	α	7.2 h	60 μm	6
^{213}Bismuth	α	46 min	84 μm	8
^{125}Iodine	Auger	60.5 d	<100 nm	(1)
^{111}Indium	Auger	3.0 d	<100 nm	(1)

*Assuming a tumor cell is 10 μm in diameter.

tumor.[116] As a consequence of their more rapid blood clearance, the fraction of the injected activity delivered to the tumor is lower with an antibody fragment than with an IgG.

Multistep *pretargeting* methods, such as those using bispecific antibodies, represent a promising method for imaging and therapy (Figure 4).[117] In this strategy, the bispecific antibody has one arm that binds to the tumor antigen while the second binds to a hapten that is typically incorporated in a small peptide that can be radiolabeled. The unlabeled bispecific antibody is first given time to circulate and bind to the tumor, and once it has cleared from the blood, the radiolabeled peptide is given. The small sized radiolabeled peptide escapes from the vasculature very rapidly, where it can bind to the other arm of the bispecific antibody on the tumor. Within minutes, the rest of the peptide clears from the blood, leaving behind only the peptide that localizes to the bispecific antibody bound to the tumor. This method has been shown in preclinical testing to improve tumor/blood ratios by as much as 40-fold, with tumor uptake increased by as much as 10-fold compared with a directly-radiolabeled antibody fragment.[118] This same method can increase the total radiation dose to tumors by 1.5-fold and increase the dose rate by 3-fold, resulting in improved antitumor responses.[119] Advances in molecular engineering have greatly enhanced the ability to provide uniform and highly novel pretargeting agents.[120,121] Other pretargeting approaches have been studied, each showing improved tumor/blood ratios, as well as improving therapy when compared with directly-radiolabeled antibodies.[117] Dosimetry data from a pilot clinical study with ^{90}Y-biotin pretargeted by a new recombinant streptavidin-anti-TAG-72 antibody are promising, and in other indications, such as medullary thyroid cancer and glioma, encouraging therapeutic results using pretargeting methods have been reported.[122–124]

Two anti-CD20 IgG-radioconjugates are currently FDA-approved for the treatment of indolent and transformed forms of NHL, ^{90}Y-ibritumomab tiuxetan (Zevalin, biogen idec) and ^{131}I-tositumomab (Glaxo SmithKline).[125] Both of these treatments improve the objective response rate compared with the unlabeled anti-CD20 antibody used to deliver the radionuclide.[101,102] Initially, there was some concern that while objective response rates were significantly improved, the pivotal trial performed with ^{90}Y-ibritumomab tiuxetan did not show a statistical improvement in the duration of the response compared with its unlabeled antibody (ie, rituximab). However, continued follow up has shown the complete responses have been more durable.[126,127] Durable responses have also been reported with ^{131}I-tositumomab, and importantly, there is evidence that when used as a front-line therapy, it is better tolerated and may improve responses compared with standard chemotherapy.[128,129] Clinical studies are also beginning to evaluate the use of ^{90}Y- ibritumomab tiuxetan as a front-line treatment and are showing these treatments do not preclude patients from receiving additional cytotoxic therapies.[130–132] Although more randomized clinical trials (RCT) and long-term follow up to assess the risk for late

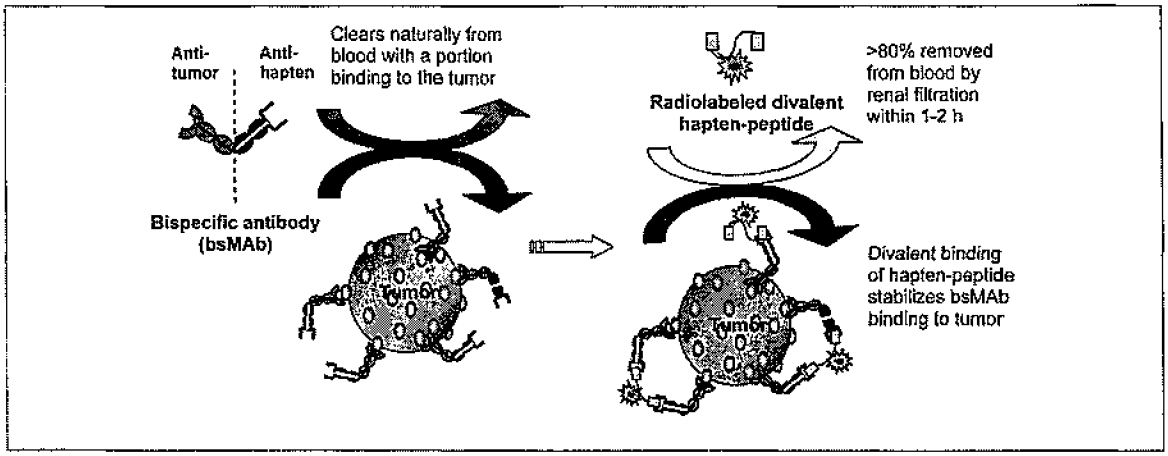

FIGURE 4 Example of a pretargeting approach using a bsMAb that has one arm capable of binding to a tumor antigen and the other arm specific for a hapten. The bsMAb is allowed to localize the tumor and clear from the blood before a radiolabeled hapten-peptide is given. The radiolabeled hapten-peptide clears from the blood very quickly, leaving behind only the material bound to the tumor. Two hapten-binding sites on the peptide help stabilize the radiolabeled hapten-peptide to the tumor.

toxicities (eg, myelodysplasia) are needed, it is impressive that a single treatment with a radiolabeled antibody with fewer side effects than the chemotherapy that is given over several months can provide such a significant benefit.[133] New efforts are underway to explore the use of these agents in other clinical indications, and new radioconjugates are being examined in lymphoma and leukemia.[112,134–138]

The application of RAIT to other tumors is considerably more challenging. The higher radioresistance of solid tumors most certainly is the primary reason why RAIT has not been as successful for these tumors, since the targeting of a variety of solid tumors is as good, if not better, than that seen in lymphoma. Despite efforts to increase the administered radiation dose by using bone marrow or peripheral stem cell support, and even by combining high-dose radioimmunotherapy with chemotherapy, clinically significant antitumor responses in solid tumors remain elusive.[108] A Phase III trial in lung cancer has indicated some success in advanced disease, but for the most part, as first emphasized in animal model testing, RAIT is more likely to succeed when the disease burden is minimal or when used as an adjuvant treatment.[139–141] Early clinical studies appear to corroborate these preclinical findings, at least in colorectal cancer, where RAIT post salvage resection of colorectal liver metastases indicated a doubling of the survival time compared with historical or contemporaneous controls.[142] Additionally, clinical studies are applying radiolabeled antibodies for intracompartmental treatments, such as intracranial and intraperitoneal therapies, where it may be possible to increase the accessibility and amount of antibody targeted to tumors in these regional areas.[143–145] Preclinical studies have shown that nontherapeutic doses of chemotherapy can enhance the effects of RAIT, while other studies have shown that relatively small doses of radiolabeled antibodies can enhance the therapeutic activity of a standard chemotherapy regimen.[146–151] The reduced hematological toxicity associated with pretargeting approaches should allow radioconjugates to be combined more readily with cytotoxic drugs.[152,153] In addition, combinations with unconjugated antibodies, such as cetuximab that can enhance the tumor's radiosensitivity, may be another option for treating EGFR-positive tumors.[154] Thus, while challenges remain for antibody-targeted radionuclides in solid tumors, preclinical and initial clinical studies are encouraging.

Drug Immunoconjugates

In the late 1950s, Mathé, et al. linked methotrexate to the globulin fraction of a hamster antiserum directed against the mouse

leukemia L1210 cell line to protect mice from subsequent inoculation with L1210 cells, providing the first evidence that antibodies could be used to target drugs.[155] As with radioconjugates, clinical success was first achieved in a hematological malignancy, with the FDA approving in 2000, gemtuzumab ozogamicin (Mylotarg; Wyeth Ayerst) for the treatment of relapsed acute myelocytic leukemia in adults (≥60 years of age).[156] Gemtuzumab ozogamicin is a conjugate of a humanized anti-CD33 IgG linked to colicheamicin, a potent antitumor agent isolated from a bacterium. The prospects of using it as a front-line treatment and expanding its indications to include pediatric cancer patients, and in combination with chemotherapy, are under evaluation.[157–160] Aside from the standard precautions for side effects associated with its infusion, other primary side effects include complications associated with severe hematological toxicity, mucositis, as well as hepatotoxicity (hyperbilirubinemia, elevated ALT, AST, and bilirubin).

Conjugation of a drug to an antibody alters the drug's pharmacodynamics, essentially "detoxifying" it, and this has allowed drugs that otherwise would be too toxic for human use alone (ie, *ultratoxic drugs*) to be tested as antibody-drug conjugates. Current clinical trials with drug conjugates almost exclusively use drugs that are far more potent than most chemotherapeutic agents, and other highly potent agents also are under development.[161–167]

The union of a biologic (antibody) and a drug (a chemical) must be made chemically, with the conjugate retaining the binding activity of the antibody, as well as the biological activity of the drug (Figure 4). Drugs may be coupled directly to an antibody or to inert carriers, such as dextrans or amino acid polymers, which have been used to increase the drug-substitution level of the conjugate.[161,162] Responses are dose-dependent, and therefore, optimizing the drug-antibody substitution level will improve the chances for success. However, a careful balance between maximizing the drug payload and maintaining favorable pharmacokinetic and biodistribution properties must be achieved.

Leukemias are a particularly attractive target for immunoconjugate therapy since the individual cells are readily available in the bloodstream and marrow. Drugs must get inside the cell to be active, and therefore, a target that is actively internalized would be more important than the target's relative abundance. For example, MAbs against CD74, which is found in low density on B-cells, monocytes, lymphomas, myelomas, and certain carcinomas, have been reported to be highly efficient carriers for drugs, toxins, and radionuclides because CD74 is readily recycled.[168–171] However, gemtuzumab ozogamicin is active even in CD33-negative cell lines because these cells are highly endocytic, and therefore, the conjugate can be internalized without specifically binding to the cell.[172] When internalized, the drug must be liberated from the antibody to regain its activity. Separation of the drug from the antibody generally occurs in the lysosomes. Ineffective trafficking and drug separation inside the cell can have a profound impact on the potency of the conjugate. Often, drugs are coupled to antibody using linkages that can only be cleaved in the acidic milieu of the lysosomes.[161,163] There were hopes that antibody-drug conjugates might overcome drug resistance by bypassing the P-glycoprotein mechanism for extruding drugs.[173] Unfortunately, this has not been realized, but one study has suggested that this might be possible under certain circumstances.[174–176]

Pretargeting approaches also have been applied to drugs. Most often referred to as ADEPT (antibody-directed enzyme prodrug therapy), this strategy first targets an antibody-enzyme conjugate to the tumor.[177] Once the conjugate is sufficiently cleared from the blood, a prodrug, which is not biologically active, is given. The prodrug is converted to an active form and released from the enzyme-conjugate. Enzymatic conversion of the prodrug continues, resulting in locally increased levels of the active drug. The ADEPT method has been tested extensively in preclinical models, as well as in early Phase I clinical studies, which initially identified the immunogenicity and clearance of the antibody-

enzyme conjugate as obstacles, but preclinical studies suggest that these problems may be overcome in the near future.[178–180]

While there are still a number of challenges to be met, new agents are being developed that will likely lead to expanded clinical evaluation of drug immunoconjugates.

Toxin Immunoconjugates

Except for denileukin diftitox (Ligand Pharmaceuticals), which is a modified diphtheria toxin coupled to interleukin-2 for the treatment of cutaneous T-cell lymphoma, no other immunotoxins have been approved by the FDA; however, there have been a number of clinical trials with a variety of toxins conjugated to antibodies.[181–183]

Toxins are truly ultratoxic agents, requiring relatively few copies to kill the cell, but they face the same delivery issues as a drug conjugate. Immunotoxins have been produced primarily from toxins that are ribosomal inactivating proteins, interfering with the reading of mRNA and thereby disrupting protein synthesis.[182] Most are natural proteins derived from plants, bacteria, or fungi, but RNases isolated from vertebrates are also being examined.[184] Since toxins have their own means for binding to cells, the cell-binding portion must be separated from the active portion of the toxin to improve targeting specificity (Figure 4).[185] As proteins, toxins are amenable to recombinant production as antibody- (or other biological targeting substance, such as interleukin-2) toxin fusion proteins.[182,183] However, toxins are foreign proteins, and therefore the formation of neutralizing antibodies is a concern for repeated use. The possible exception is RNase, which may be less immunogenic.[186]

Therapy of B-cell lymphoma using ricin A-chain conjugates prepared chemically with deglycosylated ricin A-chain and either an anti-CD19 or an anti-CD22 murine IgG was limited by the development of vascular leak syndrome (consisting of edema, tachycardia, dyspnea, weakness, and myalgia).[187–189] Recent insights into the molecular structure of the active ricin A- chain have revealed a motif that is responsible for binding to endothelial cells, which could be an important determinant in the development of dose-limiting vascular leak syndrome.[190]

A recombinant anti-CD22 x *Pseudomonas* exotoxin has been highly effective in patients with hairy cell leukemia, while not being as active in NHL CLL.[191] In hairy cell leukemia, clinical benefit (86% CR rate with a median duration of 36 months) was observed after a single cycle of conjugate treatment at a dose level of 40 μg/kg every other day x 3, with the most common toxicities being hypoalbuminemia, transaminase elevations, fatigue, and edema; a reversible hemolytic uremic syndrome requiring plasmapheresis also was observed in several patients. This conjugate's activity in hairy cell leukemia and with manageable toxicity is an exciting new development for immunotoxin conjugates.

Similar to the experience with other immunoconjugates, solid tumors remain a formidable challenge for therapy with immunotoxins. An immunotoxin prepared as a recombinant *Pseudomonas* exotoxin x anti-Lewis-Y antibody (BR96) was tested in 46 patients with Lewis-Y-positive tumors, with no objective responses reported.[192] The dose of this conjugate was limited by gastrointestinal toxicity, likely because BR96 is cross-reactive with normal gastrointestinal epithelium.[193]

ECONOMIC CONSIDERATIONS

One lesson learned from this review is that the new biological agents, particularly the unconjugated MAbs, are more effective when used in combination with other therapeutic agents, including perhaps other antibodies. Since not all patients are responsive, presumably because of differences in the receptors being targeted, molecular testing will become part of the paradigm of biological therapy to choose drugs on an individual patient basis.

But these considerations can have staggering financial implications. If the average monthly price is $4,800 for bevacizumab and $12,000 for cetuximab, combinations of these together with drugs in colorectal cancer treatment can

range between $11,000 and $27,000 monthly, along with pharmacy and dispensing costs. Since these can be prescribed over several months, the costs can challenge the healthcare system and third-party payers, as cautioned recently by Wittes.[194]

CONCLUSIONS

Antibodies and immunoconjugates are gaining a significant and expanding role in the therapy of cancer. Because patients generally tolerate antibody treatments with minimal side effects, compared with many other cancer treatment modalities, immunotherapy with antibodies represents an exciting opportunity for combining with standard modalities, such as chemotherapy, as well as combinations between diverse biological agents, including antibody combinations in NHL therapy and possibly cetuximab + bevacizumab (with chemotherapy) in metastatic colorectal cancer.[195] As we learn more about how cancer and other diseased cells control their proliferation and spread, undoubtedly unconjugated antibodies will be used to disrupt these functions by targeting important sites or regulators of cell proliferation, metabolism, adhesion, migration, spread, and other properties of malignancy. The use of antibodies to target radionuclides, drugs, and toxins is expanding as the next generation of MAb-based products for cancer therapy. At least in the case of targeted radionuclides, clinical studies have shown that these immunoconjugates are more effective than immunotherapy with the antibody alone, which highlights the enhanced efficacy achieved when a cytotoxic agent is targeted by an antibody that is also active.

This review has summarized the strides made over the past 25 years for developing new, selective, therapeutic strategies based on the evolution of various antibody forms and an identification of new cellular targets. Molecular biology has been at the basis of developing this new generation of antigen-binding molecules. As new target molecules and receptors on tumor cells are identified in the future, the experiences gained with the use of current immunoconjugates will enable a more rapid translation to clinical evaluation and use when next-generation antibodies and immunoconjugates are developed.

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Research Article

Tumor Cell–Secreted Caveolin-1 Has Proangiogenic Activities in Prostate Cancer

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Abstract

Caveolin, a major structural component of specialized plasma membrane invaginations (caveolae) that participate in diverse cell activities, has been implicated in the pathogenesis of several human diseases, including cancer. We showed in earlier studies that caveolin-1 (cav-1) is consistently and strongly overexpressed in metastatic prostate cancer and is secreted in a biologically active form by virulent prostate cancer cells. Using both *in vitro* and *in vivo* model systems, we now present evidence supporting a proangiogenic role for cav-1 in prostate tumor development and progression. Recombinant cav-1 (rcav-1) was taken up by cav-1$^{-/-}$ tumor–associated endothelial cells through either a lipid raft/caveolae- or clathrin-dependent mechanism, leading to specific angiogenic activities (tubule formation, cell migration, and nitric oxide production) that were mediated by rcav-1 stimulation of the PI3K-Akt-eNOS signaling module. Pathologic angiogenesis induced by cav-1 in prostate tumor–bearing mice correlated with an increased frequency, number, and size of lung metastases. We propose that in addition to its antiapoptotic role, cav-1 secreted by prostate cancer cells functions critically as a proangiogenic factor in metastatic progression of this tumor. These new insights into cav-1 function in prostate cancer may provide a base for the design of clinically applicable therapeutic strategies. [Cancer Res 2008;68(3):1–9]

Introduction

As essential components of caveolae, caveolin proteins help to generate and maintain these highly ordered structures at the cell surface. They also mediated endocytosis and transcytosis of molecules attached to the cell surface and organize signaling proteins involved in cell proliferation, adhesion, and migration, among numerous other biological processes (1). This functional versatility has focused increasing attention on the possible role of caveolins in cancer development and progression. Findings to date clearly indicate that caveolin-1 (cav-1), the first of several caveolin family members that differ in structure and tissue distribution, can influence both tumorigenesis and metastatic spread in certain types of cancer (2–6), although the mechanisms of these effects are largely unknown. We showed in earlier studies that cav-1 is consistently and strongly overexpressed in metastatic prostate cancer and is secreted in a biologically active form by virulent prostate cancer cells (2, 3, 7). Interestingly, we detected significantly increased serum cav-1 levels in prostate cancer patients compared with control men or men with benign prostatic hyperplasia, and showed that preoperative serum cav-1 is a potential prognostic marker for recurrence in radical prostatectomy cohort (8, 9). The ability of some prostate cancer cells to secrete biologically active cav-1 (7, 8), and the demonstration that loss of cav-1 function in the TRAMP transgenic mouse prostate cancer model results in highly significant reductions of prostate cancer growth and metastasis (10), led us to suspect that tumor cell–secreted cav-1 may function as a paracrine factor during prostate cancer development, possibly as a regulator of pathologic angiogenesis. The studies described here substantiate this role and suggest a paradigm that may be applicable to other tumors that secrete cav-1.

Materials and Methods

Endothelial cell isolation. Endothelial cells from *cav-1*$^{-/-}$ mice (11) were isolated from mouse aorta according to the primary explant procedure and used throughout the study. Briefly, the aorta was removed from the anaesthetized mice, placed in PBS, and carefully cleaned of periadventitial fat and connective tissue. The vessel was then cut into 1-mm pieces, opened longitudinally, and placed with the intima side down on Matrigel-coated (BD Biosciences) 12-well plates in endothelial cell growth medium (EGM; Cambrex) to generate endothelial outgrowth. The aortic pieces were removed after 4 to 7 days, and the cells were allowed to grow to confluence. After recovery with dispase, the cells were plated on a 12-well plate and then subcultured twice. The confluent monolayers showed the typical cobblestone pattern of endothelial cells stained positively for uptake of Di-I-Ac-LDL (Biomedical Technologies).

Western blotting. Protein aliquots from cell lysates were separated by 10% or 12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with antibodies to cav-1 (Santa Cruz Biotechnology), eNOS, Erk1/2, Akt (BD Biosciences), P-Akt, P-eNOS, or P-Erk1/2 (Cell Signaling Technology).

Recombinant cav-1 and Δrecombinant cav-1 purification. phCav-1V5 and phΔcav-1V5His plasmids were constructed as described previously (8), whereas recombinant cav-1 (rcav-1) and Δrcav-1 were purified by our modified procedure. Briefly, transfected 293PE cells were washed with PBS and lysed with 10 mL of ice-cold buffer A [50 mmol/L phosphate buffer, 300 mmol/L NaCl, 10 mmol/L imidazole, and 5 mmol/L mercaptoethanol (pH.8)] containing 0.5% Triton X-100 and 0.7% octylβ-D-glucopyranoside (OGP). The lysate was centrifuged for 15 min at 4°C, 12,000 × *g*, and the supernatant was mixed and incubated with 1 mL of Ni-NTA agarose slurry for 3 h. The resultant mixture was loaded on to a 10 mL polyprep column (Bio-Rad), and the resin was washed with 10 volumes of buffer A containing 500 mmol/L NaCl, 50 mmol/L imidazole, and 0.2% OGP. The bound cav-1-V5-His was eluted with 3 mL of elution buffer (buffer A containing 300 mmol/L imidazole, 300 mmol/L NaCl, and 0.1% OGP). For Western blot analysis, the crude supernatant as well as unbound and eluted fractions were subjected to SDS-PAGE. FITC labeling of recombinant cav-1 proteins was prepared with the EZ-label FITC

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EXHIBIT 3

protein labeling kit (Pierce Biotechnology, Inc.) according to the manufacturer's instructions.

Tubule formation assay. The *in vitro* tubule formation assay was used as described previously (12). Briefly, endothelial cells were incubated in growth factor–reduced Matrigel-coated 24-well plates in 0.5 mL of endothelial basement medium (EBM; Cambrex) in the presence or absence of rcav-1 or Δrcav-1. Images of tubule structures that formed after 18 to 24 h were captured by phase contrast microscopy, and the length of the endothelial network was quantified by image analysis of five low-power fields using free object quantification software (NucleoTech Corp.).

Wound-healing migration assay. Endothelial cells were cultured in 24-well plates to 70% to 80% confluency in EGM, and a straight longitudinal incision was made on the monolayer. After a wash with EBM and incubation with rcav-1 or Δrcav-1 in EBM containing 0.1% bovine serum albumin (BSA) for 4 h followed by an additional 48 h of incubation in EBM containing 2% of fetal bovine serum (FBS), the cells were stained with the Protocol HEMA3 stain set (Biochemical Sciences, Inc.), and the number of cells migrating into the cleared area were counted with a microscope, using advanced colony counting software (NucleoTech Corp.).

Cell proliferation and [^{3}H]-thymidine incorporation. Endothelial cells were seeded into 12-well plates (5×10^4 cells per well) and incubated overnight. After the medium was removed, the cells were treated with rcav-1 in EBM for 4 h and incubated for an additional 48 h in EBM containing 2% FBS, after which they were trypsinized and counted with a coulter counter. For [^{3}H]-thymidine uptake, the endothelial cells were seeded into 96-well plates (2.5×10^3 cells per well) in EGM then treated with rcav-1 and incubated for 48 h in EGM. [^{3}H]-thymidine (5 μCi/mL) was then added, the cells were incubated for 24 h, and the cell lysate-associated radioactivity was counted.

Nitric oxide determination. The basal and rcav-1 stimulated NO derived from endothelial cells that had accumulated in EBM over a 24-h period was measured with the Nitric Oxide Colorimetric Assay (Roche Diagnostics).

PP1 and PP2A activities. Endothelial cells were treated with rcav-1 and incubated in EBM containing 0.1% BSA for 24 h at 37°C and 5.5% CO_2. The cells were lysed with ice-cold phosphatase lysis buffer, and PP1 and PP2A activities were measured after immunoprecipitation as described previously (13).

Animal models. Orthotopic RM-9 tumors were generated by injecting 5×10^3 cells directly into the dorsolateral prostates of *cav-1*$^{+/+}$ or *cav-1*$^{-/-}$ male mice. The resultant tumors were removed at necroscopy on day 21 postinjection, and their wet weight were determined; all tumors were processed for specific immunostaining protocols (see below).

To generate the LNCaP cav-1 tet-on system, we transfected *cav-1*$^{-/-}$ low passage (LP)-LNCaP cells with pTetOn vector (Clontech), isolated stable G418-resistant clones, and screened them in a transient trasfection reporter assay with pTRE2Luc vector according to the manufacturer's protocol with or without 1 μg/mL doxycycline. Clone LNT36, which had the highest induction level, was chosen for the second cotransfection, in which a pTRECav-1 vector containing full-length human *cav-1* cDNA and the pBabeHygro plasmid. Double stable G418- and hygromycin-resistant clones were isolated and tested for cav-1 induction in response to the doxycycline (1.0 μg/mL). Clone LNTB25cav, which showed strong induction of cav-1 after addition of doxycycline to the medium and the lowest endogenous expression in the absence of the drug *in vitro*, was used for further *in vivo* studies.

To establish xenografts, we inoculated male nude mice with LNTB25cav cells that were suspended in Matrigel matrix and injected s.c. Tumors were present 21 days after inoculation, and tumor-bearing mice were divided into two groups that were normalized for tumor size. One group was treated with drinking water containing doxycycline (2 mg/mL) and 5% sucrose, whereas the other (control group) was treated with drinking water containing only 5% sucrose. After 21 days, the animals were sacrificed, and the tumor tissues were harvested and either snap frozen in liquid nitrogen or fixed in 10% neutral formalin.

For the *in vivo* metastasis assay, 1×10^6 LNTB25cav cells were injected into the tail veins of male nude mice to establish experimental metastases. Two months after the initial injection, the mice were divided into two groups: one was treated with drinking water containing doxycycline (2 mg/mL) and 5% sucrose and the other (control group) with drinking water containing only 5% sucrose. After a 42-day treatment, the animals were sacrificed and lung tissue was collected, fixed, and analyzed for tumor foci.

Immunohistochemistry and deconvolution microscopy. Depending on the fluorescent protein treatment, LNCaP, PC-3, and TSU-Pr1 tumor cells or endothelial cells were placed on glass coverslips in 24-well plates and incubated overnight in RPMI 1640 or EGM, respectively. After removal of the medium, the cells were washed twice with PBS buffer, then FITC-rcav-1, FITC-Δrcav-1, Alexa fluor 594–labeled cholera toxin B, and transferrin (Invitrogen) were added to medium that contained 0.1% BSA. The cells were incubated for 5 h, rinsed twice with PBS buffer, and fixed in 4% formaldehyde for 5 min at room temperature.

For immunostaining, fixed cells were permeabilized with 0.1% Triton X-100 in PBS buffer and blocked with 3% normal horse or goat serum. They were then incubated with primary antibody followed by biotinylated anti-rabbit IgG (Vector Labs) and rhodamine-conjugated streptavidin or FITC-streptavidin (Jackson Immuno Research). Reactions were evaluated with the Delta Vision Deconvolution Microscopy System (Applied Precision, Inc.), in which a Z-series of optical sections (0.15-μm steps) were digitally imaged and deconvolved with the Delta Vision–constrained iterative algorithm to generate high-resolution images.

Mouse model–derived tumor specimens were stained for CD31 (BD Biosciences) using the avidin-biotin-peroxidase complex technique (ABC kit; Vector Lab) as previously described (14). Quantitative analysis of microvessel density was performed on the stained sections. The vascular "hot region" was first identified by low-power screening (magnification, ×40). Vascular counting was then performed on at least five 200× measuring fields (each with a real area of 0.198 mm^2). For each sample, the highest count per field was used.

Dual-immunofluorescence staining was also performed on these tissues. Briefly, after tissue sections were deparaffinized and rehydrated through graded alcohol, they were heated in 0.01 mol/L citrate buffer at pH 6.0 by microwave for 10 min to enhance antigen retrieval. After a 20-min blocking step with 1.5% normal goat serum, the sections were sequentially incubated with polyclonal cav-1 antibody diluted 1:200 for 90 min, followed by biotinylated anti-rabbit IgG and streptavidin-FITC for 30 min each. The sections were reblocked in 1.5% normal horse serum for 20 min and incubated in CD31 monoclonal antibody followed by Cy-3–conjugated anti-rat IgG for mouse specimens. The specificity of immunoreactions was verified by replacing the primary antibodies with PBS or with corresponding normal serum. The labeled specimens were evaluated using a Zeiss fluorescence microscope equipped with a video camera (Hamamatsu). Each section was analyzed systematically, field-by-field (300×400 μm^2), over the area of cancer cells. The percentages of cav-1–positive CD31 microvessels were determined for each field for each fluorophore and on superimposed images of both fluorophores with the aid of OPTIMAS (6.0) software.

Statistical analysis. The Mann-Whitney rank test was used to analyze differences in microvessel density within mouse prostate cancer tissues; comparisons of *in vitro* tubule formation, cell migration, phosphatase activity assay, NO release assay, and RM-9 tumor wet weights relied on the unpaired two-sided *t* test. Fisher's exact test was used for the comparison of the metastasis frequency in LNTB25cav-injected mice. All statistical analyses were performed with Statview software (Version 5.0; SAS Institute).

Results

Cav-1 uptake by prostate cancer cells and endothelial cells. We have shown that prostate cancer cells secrete cav-1 possessing antiapoptotic activity that can be suppressed by cav-1–specific antiserum *in vitro* (7). Such antiserum also suppressed metastasis *in vivo*, raising the possibility that secreted cav-1 is taken up by tumor cells or tumor-associated endothelial cells or both. Thus, we treated cav-1–negative LP-LNCaP tumor cells or primary

endothelial cells, isolated from *cav-1*$^{-/-}$ mouse aorta, with conditioned medium collected from cav-1–transfected LP-LNCaP cells or with rcav-1 alone. Western blot analysis showed that cav-1 contained in contained medium was taken up by LP-LNCaP cells in a dose- and time-dependent manner, as indicated by the appearance of cav-1 in cell lysates within 1 h and the achievement of maximal intracellular levels 3 h posttreatment (Fig. 1*A*). Rcav-1 protein was also taken up by the LP-LNCaP cells and *cav-1*$^{-/-}$ endothelial cells in a dose-dependent fashion over a 24-h incubation period (Fig. 1*B* and *C*). Rcav-1 uptake by tumor cells (LP-LNCaP, TSU-Pr1, and PC-3) and endothelial cells [human umbilical vascular endothelial cell (HUVEC), and mouse *cav-1*$^{-/-}$ and *cav-1*$^{+/+}$] was further shown by fluorescence and deconvolution microscopy. FITC-rcav-1 uptake by these cells was temperature dependent, with 5 h of incubation at 0°C, abolishing uptake altogether (data not shown). Internalized FITC-rcav-1 was distributed throughout the cytoplasm (Fig. 1*D*).

F1

Lipid raft/caveolae-dependent and clathrin-dependent endocytic pathways are involved in rcav-1 internalization in endothelial cells. To determine the endocytic pathways responsible for rcav-1 internalization, we pretreated HUVEC and *cav-1*$^{+/+}$ or *cav-1*$^{-/-}$ mouse endothelial cells with methyl-β-cyclodextrin (MCD) or chlorpromazine to disrupt the formation of cholesterol-rich raft microdomains or clathrin-coated pits, respectively. Fluorescence microscopy revealed that MCD effectively inhibited FITC-rcav-1 uptake in both types of endothelial cells, whereas chlorpromazine inhibited FITC-rcav-1 uptake effectively in mouse endothelial cells but only marginally in HUVEC (Fig. 2*A*). Under the same conditions, MCD effectively reduced the uptake of cholera toxin B, whereas chlorpromazine reduced the uptake of transferrin substances known to penetrate cells through cholesterol-rich lipid raft and clathrin endocytic pathways, respectively (Fig. 2*B*). These results indicate that internalization of exogenous rcav-1 proceeds through lipid raft/caveolae and clathrin pathways in both HUVEC and mouse endothelial cells, with the former pathway dominant in HUVEC (Fig. 2*A*, *left*). To directly show that rcav-1 associates with internalized lipid rafts/caveolae to enter endothelial cells, we incubated HUVEC for 5 h with a mixture of FITC-rcav-1 and cholera toxin B and tested for their cellular colocalization. We found that a majority (76%) of the FITC-rcav-1–positive endosomes also contained cholera toxin B (Fig. 2*C*), indicative of a requirement for caveolae and ganglioside G_{M1} lipid rafts in cav-1 penetration of human endothelial cells.

F2

Internalization of rcav-1 is mediated by cav-1 scaffolding domain. Mutagenesis experiments have identified cav-1 scaffolding domain (CSD) residues 82 to 101 as the region responsible for mediating interactions with a number of signaling proteins including the endothelial form of nitric oxide synthase (eNOS), platelet-activating factor receptors, epidermal growth factor, the kinases Src and Fyn, heterotrimeric G protein,

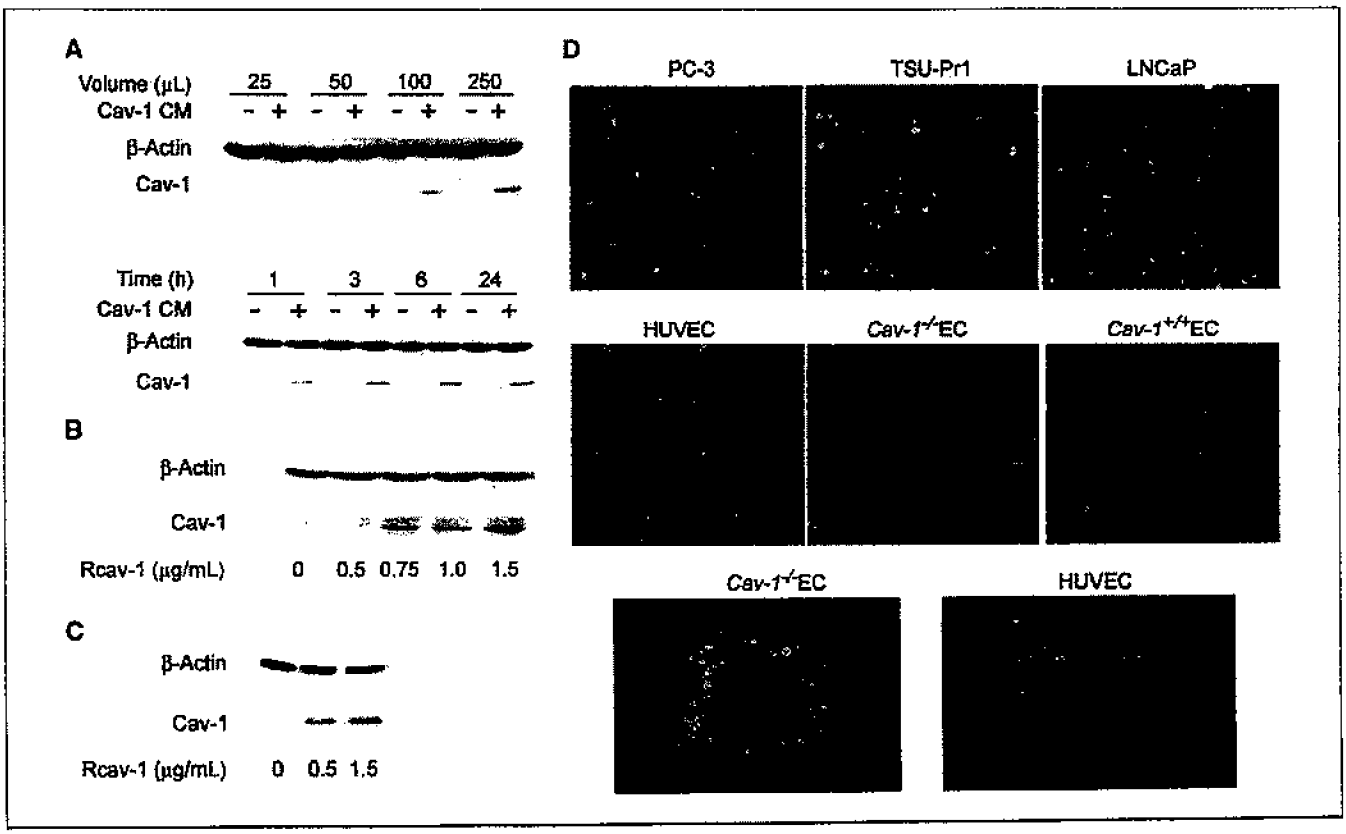

Figure 1. Cav-1 uptake by prostate cancer and bladder cancer cells and endothelial cells. *A*, dose- and time-dependent uptake of cav-1 from *cav-1*–transfected (+) or control-transfected (−) contained medium (*CM*) by LP-LNCaP cells. *Top*, detection of cav-1 after a 24-h treatment with contained medium over a range of volumes; *bottom*, detection after 1 to 24 h of treatment with 250 μL contained medium. *B* and *C*, dose-dependent rcav-1 uptake by LP-LNCaP tumor cells (*B*) and *cav-1*$^{-/-}$ endothelial cells (*EC*; *C*) treated for 24 h. *D*, internalization of FITC-rcav-1 by cancer cells (*top*) and endothelial cells (*middle*) treated with 3.0 μg/mL of FITC-rcav-1 for 5 h. Uptake by endothelial cells (*cav-1*$^{-/-}$ endothelial cells and HUVEC) were imaged by deconvolution microscopy after treatment with FITC-rcav-1 (*bottom*); nuclei were visualized by Hoechst 33342 staining.

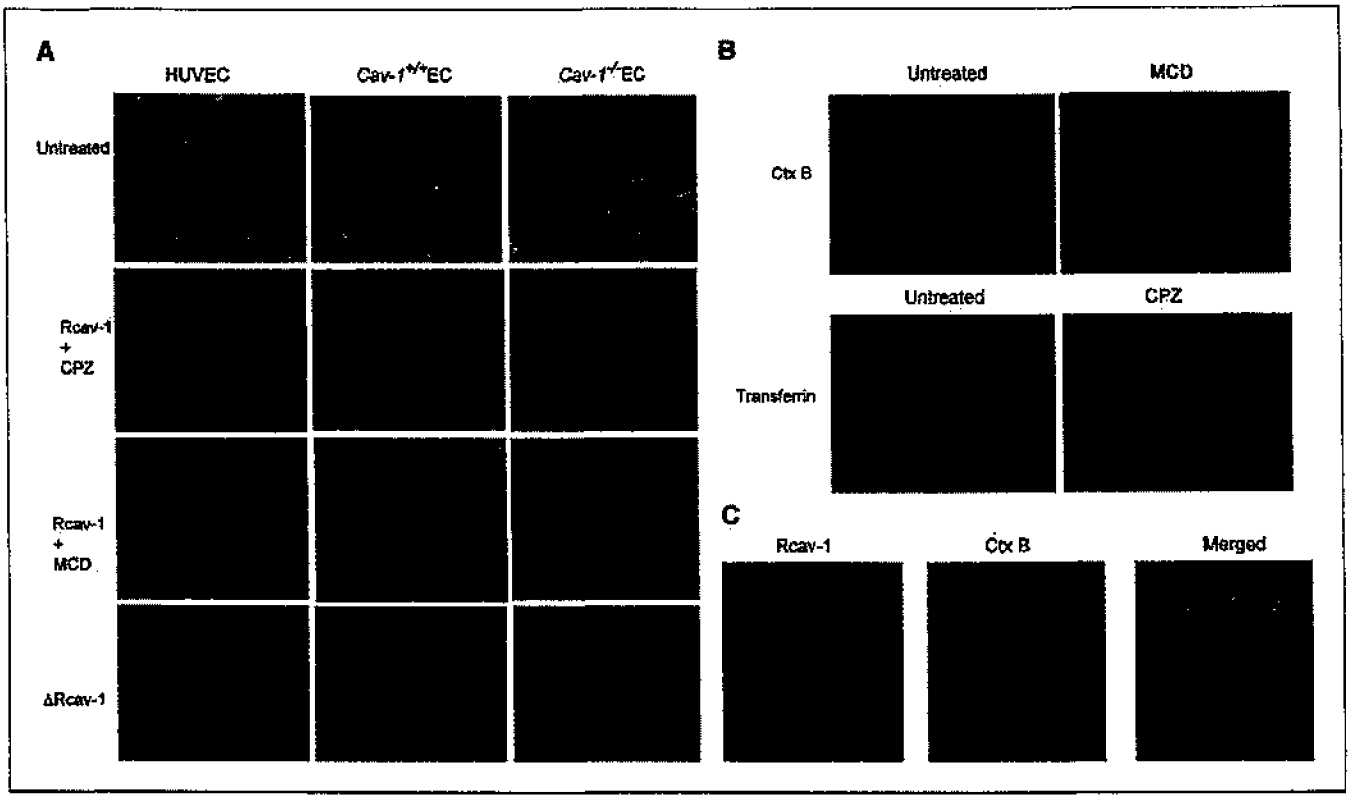

Figure 2. Internalization of rcav-1 by lipid raft/caveolae-dependent and clathrin-dependent endocytic pathways. *A*, cells were incubated with FITC-rcav-1 (3.0 μg/mL) in the presence or absence of 7.5 μg/mL of chlorpromazine (*CPZ*) or 7 mmol/L MCD for 5 h and analyzed by fluorescence microscopy. *B*, cholera toxin B (*Ctx B*) and transferrin internalization are blocked by MCD and chlorpromazine, respectively. HUVEC cells were incubated with Alexa fluor 594–labeled cholera toxin B and transferrin containing the same MCD and chlorpromazine concentrations as in *A* for 5 h and analyzed by fluorescence microscopy. Cholera toxin B internalization was impaired by cholesterol depletion (MCD treatment), whereas transferring uptake was blocked by disruption of clathrin-coated pits (chlorpromazine treatment). *C*, colocalization of internalized FITC-rcav-1 with cholera toxin B, a ganglioside G_{M1} lipid raft/caveolae marker, as detected by deconvolution microscopy of HUVEC cells after the incubation for 5 h with FITC-rcav-1 and Alexa fluor 594–labeled cholera toxin B; nuclei were visualized by Hoechst 33342 staining.

and cholesterol-binding protein (15). This domain also targets the full-length endogenous cav-1 to lipid rafts/caveolae and cell membranes (16). To determine the role of the CSD in exogenous rcav-1 membrane attachment and cellular uptake, we generated and purified the CSD-deleted rcav-1 protein (Δrcav-1), treated endothelial cells and prostate cancer cells with different concentrations of FITC-Δrcav-1 over 1 to 6 h, and examined the cells for Δrcav-1 uptake using fluorescence microscopy. We did not detect internalized FITC-Δrcav-1 in cells incubated for as long as 6 h at concentrations of the mutant protein ranging to 5.0 μg/mL (Fig. 2*A*). In separate coincubation experiments, we showed uptake of cholera toxin B or transferrin under the same conditions (data not shown). These observations suggest that endocytosis of exogenous rcav-1 protein and its subsequent stimulation of angiogenic activities is mediated, in part, by CSD, which seems critical for cellular internalization of the protein.

Rcav-1 stimulates differentiation and migration of *cav-1*$^{-/-}$ endothelial cells. We initially analyzed the formation of tubules by endothelial cells, isolated from *cav-1*$^{+/+}$ or *cav-1*$^{-/-}$ aorta, on growth factor–reduced Matrigel. Compared with *cav-1*$^{+/+}$ endothelial cells, cells lacking this gene showed significantly reduced tubule formation in the absence of rcav-1 stimulation (Fig. 3*A*; micrographs). However, treatment with rcav-1 stimulated tubule formation in *cav-1*$^{-/-}$ endothelial cells in a dose-dependent manner with a >2-fold increase in tubule length observed with use of 1.5 μg/mL rcav-1 compared with untreated controls (P = 0.021). Importantly, Δrcav-1 at this concentration failed to stimulate tubule formation (Fig. 3*A*). To determine the effects of rcav-1 on *cav-1*$^{-/-}$ endothelial cell migration, we used the *in vitro* wound-healing assay. Rcav-1 treatment stimulated *cav-1*$^{-/-}$ endothelial cell migration in a dose-dependent fashion with a 2-fold increase in the number of migratory cells at a rcav-1 concentration of 1.5 μg/mL (P = 0.019), whereas Δrcav-1 at this concentration failed to increase migration/motility of the endothelial cells (Fig. 3*B*). This enhancement of tubule formation and the number of migratory/motile cells by rcav-1 treatment did not result from increased cell proliferation, as the numbers of cells or levels of thymidine uptake posttreatment were similar to the results for untreated controls (data not shown).

Rcav-1 stimulates the angiogenic activities in *cav-1*$^{-/-}$ endothelial cells through the activation of eNOS. Caveolae and cav-1 play critical roles in ensuring the coupling between vascular endothelial growth factor (VEGF) receptors and downstream mediators of angiogenesis, such as VEGF, which activates Erk and eNOS via the phosphatidylinositol-3-kinase (PI3-K)-Akt signaling pathway (17–19). Thus, to assess the contribution of this signaling module to the angiogenic activities of rcav-1, we tested the effects of inhibitors of PI3 kinase (LY294002), eNOS (L-NAME), and Erk (PD98059) in *cav-1*$^{-/-}$ endothelial cells. Figure 3*C* and *D* shows that both LY294002 and L-NAME, but not PD98059, significantly suppressed rcav-1–stimulated angiogenesis, implicating PI3-K-Akt-eNOS signaling in the pathologic angiogenic effects

of cav-1 in prostate cancer cells. To investigate this possibility further, we measured the levels of accumulated NO (NO_2^- + NO_3^-) at 24 h after rcav-1 treatment of *cav-1*$^{-/-}$ endothelial cells. NO release by these cells was significantly increased by rcav-1 in a dose-dependent manner (P = 0.029 versus untreated control; Fig. 4*A*, *left*). Analysis of the effects of rcav-1 on the phosphorylation status of Akt and its downstream target protein eNOS in *cav-1*$^{-/-}$ endothelial cells showed a dose-dependent increase in Akt phosphorylation on S473 and T308 with no change in total Akt. Rcav-1 treatment also led to increased eNOS phosphorylation on S1177 but not T495 (Fig. 4*A*, *right*). The CSD-deleted rcav-1 failed to stimulate eNOS S1177 phosphorylation, as expected (Fig. 4*B*, *top*). We also tested the effect of LY294002 on the rcav-1–induced phosphorylation of Akt (T308) and eNOS (S1177) in *cav-1*$^{-/-}$ endothelial cells. As expected, LY294002 treatment of the cells diminished the observed Akt phosphorylation induction by rcav-1. Interestingly, the phosphorylation of eNOS (S1177) induced by rcav-1 was reduced but not completely diminished as a result of LY294002 treatment (Fig. 4*B*, *bottom*).

To further investigate the mechanism(s) that underlies rcav-1–stimulated eNOS activation, we tested the effect of rcav-1 on the activities of serine/threonine protein phosphatases PP1 and PP2A in *cav-1*$^{-/-}$ endothelial cells. These two phosphatases are known to regulate the phosphorylation of multiple protein targets including Akt and eNOS (20, 21) and are inhibited by cav-1 overexpression in prostate cancer cells (13). The activation of eNOS by a number of stimuli including VEGF involves a transient increase in the phosphorylation of S1177 with a decrease in T495 phosphorylation, alternatively, protein kinase C signaling inhibits eNOS activity by phosphorylating T495 and dephosphorylating S1177. Both PP1 and PP2A are associated with eNOS phosphorylation. PP1 is specific for dephosphorylation of T495, whereas PP2A is specific for S1177

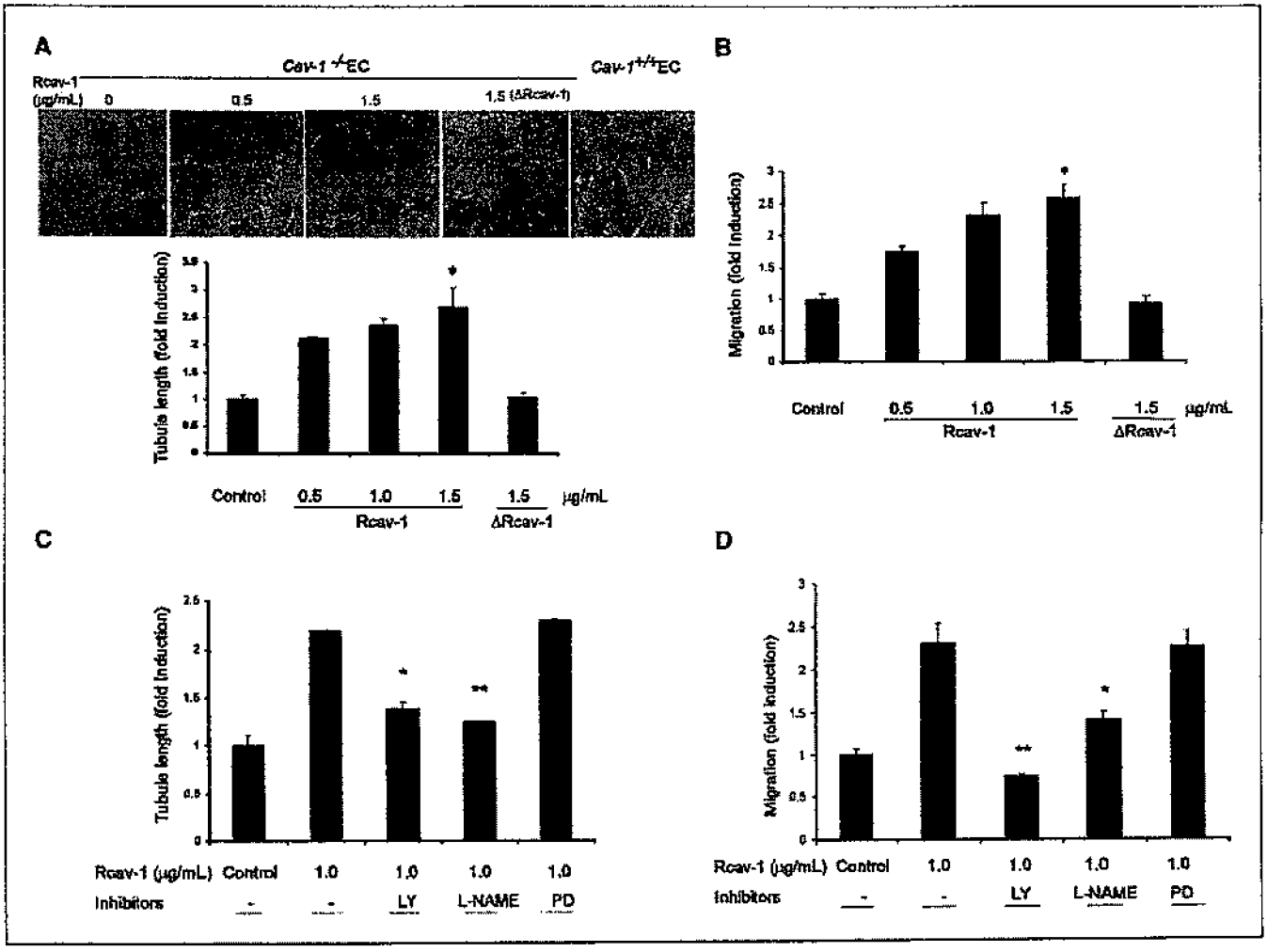

Figure 3. Rcav-1 stimulates tubule formation and cell migration in *cav-1*$^{-/-}$ endothelial cells. *A*, representative micrographs showing newly formed tubules of *cav-1*$^{+/+}$ and *cav-1*$^{-/-}$ endothelial cells cultured on growth factor–reduced Matrigel under basal conditions or after treatment with 0.5 to 1.5 μg/mL of rcav-1 and 1.5 μg/mL mL Δrcav-1 for 18 h. Bar graph depicts dose-dependent rcav-1 or Δrcav-1 stimulation of tubule formation in *cav-1*$^{-/-}$ endothelial cells. The values are folds of induction relative to untreated control ± SD of three independent experiments. *, P = 0.02 versus untreated control by two-sided t test. *B*, dose-dependent rcav-1 or Δrcav-1 stimulation of *cav-1*$^{-/-}$ endothelial cell migration in a wound-healing assay. The values are folds of induction relative to untreated control + SD of three independent experiments. *, P = 0.0193 versus untreated control by two-sided t test. *C*, inhibition of rcav-1–stimulated tubule formation by LY294002 (*LY*; 3.0 μmol/L) or L-NAME (1.0 mmol/L) but not by PD98059 (*PD*; 50 μmol/L) in *cav-1*$^{-/-}$ endothelial cells. *, P = 0.008; **, P = 0.003 versus rcav-1 treated only. *D*, inhibition of rcav-1–stimulated wound-healing assay cell migration by LY294002 (3.0 μmol/L) or L-NAME (1.0 mmol/L), but not by PD98059 (50 μmol/L) in *cav-1*$^{-/-}$ endothelial cells. *, P = 0.011; **, P = 0.005 versus rcav-1 treated only, by-two sided t test. Bar graphs in *C* and *D* represent tubule length relative to untreated controls and the number of migratory cells relative to untreated controls, respectively. *Columns*, mean; *bars*, SD.

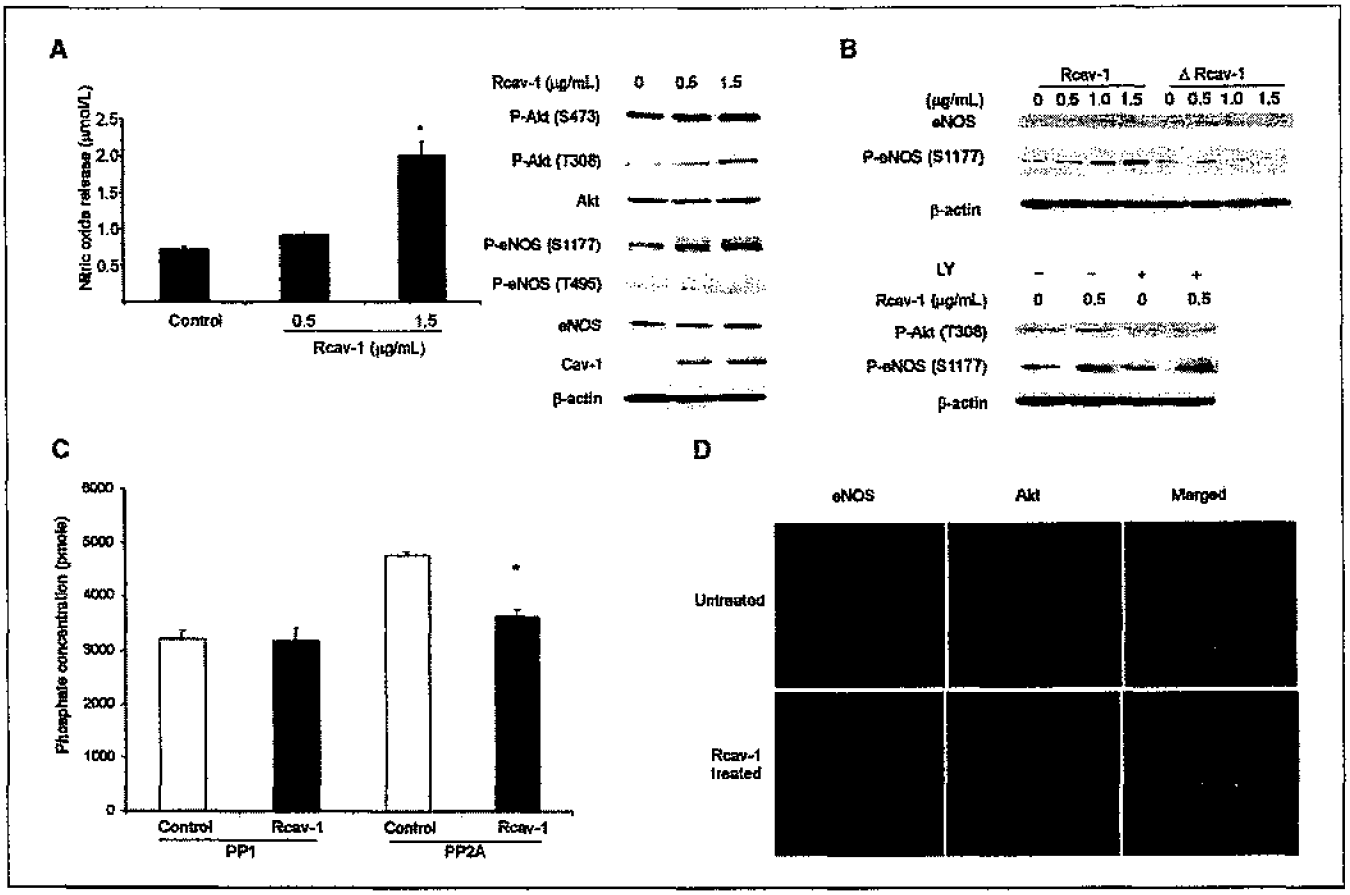

Figure 4. Rcav-1 is involved in PI3-K-Akt-eNOS–mediated stimulation of angiogenic activities in *cav-1*$^{-/-}$ endothelial cells. *A*, dose-dependent NO release by *cav-1*$^{-/-}$ endothelial cells after rcav-1 treatment. *Columns*, mean; *bars*, SD. *, $P = 0.029$ versus untreated control, by two-sided *t* test (*left*). Increased phosphorylation of Akt on S473 and T308, and of eNOS on S1177 by Western blot analysis of *cav-1*$^{-/-}$ endothelial cells lysates treated for 24 h with different concentrations of rcav-1 (*right*). *B*, Δrcav-1 treatment of *cav-1*$^{-/-}$ endothelial cells for 24 h does not affect the phosphorylation status of eNOS on S1177, but rcav-1 increases eNOS phosphorylation on S1177 in a dose-dependent fashion (*top*). Treatment of *cav-1*$^{-/-}$ endothelial cells with LY29400 abolishes the rcav-1–induced Akt phosphorylation on T308 and reduces, but not completely eliminate, the eNOS phosphorylation on S1177 induced by rcav-1 (*bottom*). *C*, rcav-1 inhibits the activity of PP2A but not PP1 in *cav-1*$^{-/-}$ endothelial cells. PP1-C or PP2A-C immunoprecipitation complexes from rcav-1–treated *cav-1*$^{-/-}$ endothelial cells or untreated controls were used to determine phosphatase activities with the serine/threonine protein phosphatase assay. *Columns*, mean; *bars*, SD. *, $P = 0.0002$ by two-sided *t* test. *D*, induction of eNOS/Akt association by rcav-1. *Cav-1*$^{-/-}$ endothelial cells were cultured for 6 h in the presence or absence of rcav-1. After fixation, the cells were double-labeled with anti-eNOS and anti-Akt immunofluorescence. In untreated cells, eNOS (*red*) and Akt (*green*) were localized to separate compartments (*top*), whereas rcav-1 protein treatment of the cells for 6 h induced eNOS (*red*) and Akt (*green*) colocalization in cytoplasmic vesicles (*bottom*), as visualized by deconvolution microscopy.

dephosphorylation (21). The results showed that rcav-1 treatment significantly inhibited the activity of PP2A but had no effect on PP1 activity ($P = 0.0002$ versus control; Fig. 4*C*). These data provide evidence that rcav-1 induces eNOS phosphorylation through Akt activation, and independently of Akt, through inhibition of PP2A, which specifically dephosphorylates eNOS (S1177).

A number of studies have shown that both eNOS and PI3 kinase are colocalized within the caveolar region of the plasma membrane (22, 23); therefore, we investigated the role played by cav-1 in compartmentalization of the PI3-K-Akt-eNOS signaling pathway molecules in *cav-1*$^{-/-}$ endothelial cells. We incubated the cells with or without rcav-1 for 5 h and visualized the cells by deconvolution microscopy for colocalization of Akt with eNOS. We found that Akt was not colocalized with eNOS in untreated cells, whereas significant colocalization of the two molecules was observed in the cells treated with rcav-1 (Fig. 4*D*).

Rcav-1 uptake in tumor-associated endothelial cells and proangiogenic activities in prostate cancer animal models. To investigate the effects of endothelial cells–localized cav-1 on microvessel density and tumor growth *in vivo*, we used an orthotopic RM-9 mouse prostate cancer model (24), in which cav-1 expressing and secreting RM-9 prostate cancer cells are injected directly into the dorsolateral prostate of male *cav-1*$^{+/+}$ or *cav-1*$^{-/-}$ mice. In this model, the mean (1.85 + 0.167) tumor wet weight was significantly higher in *cav-1*$^{+/+}$ versus *cav-1*$^{-/-}$ mice ($P = 0.045$; Fig. 5*A*). Moreover, immunohistochemical analysis of tumor sections collected from sacrificed mice showed that RM-9 tumors had significantly higher microvessel densities in *cav-1*$^{+/+}$ compared with *cav-1*$^{-/-}$ hosts [median, 21.5 (range, 15.6–36.1) versus 13.3 (range, 8.2–22.8; $P = 0.0078$); Fig. 5*B* and *C*). Interestingly, >70% of the CD31^{+} microvessels in the *cav-1*$^{-/-}$ mouse tumor sections were positive for cav-1 staining, indicating uptake of RM-9 cell–derived cav-1 by tumor-associated endothelial cells (Fig. 5*D*, *arrows*). F5

We examined the association between cav-1 expression and prostate tumor–associated angiogenesis more closely by generating an LNCaP tet-on cav-1 stable cell line (LNTB25cav) in which the expression of cav-1 can be regulated by manipulating doxycycline. In the absence of doxycycline, the level of cav-1 protein in lysate is low, whereas the addition of doxycycline to the culture medium

F6 leads to a rapid induction of cav-1 protein *in vitro* (Fig. 6*A*). LNTB25cav tumors were established as s.c. growing xenografts in adult male nude mice; tumor-bearing mice were then treated with either doxycycline or control sucrose solution added to the drinking water. Tumor volumes in the doxycycline-treated group were significantly greater than those in the control group on days 12, 15, and 18 after treatment ($P = 0.0195$, $P = 0.035$, $P = 0.019$, respectively; Fig. 6*A*). Further immunohistochemical analysis showed increased cav-1 levels in the cytoplasm of tumor cells in doxycycline-treated compared with control mice (Fig. 6*B*, *top*). Microvessel densities determined by CD31 labeling were greater in cav-1–induced tumors compared with controls ($P = 0.039$; Fig. 6*B*, *bottom*; Fig. 6*C*). In separate experiments, we injected 1×10^6 LNTB25cav cells into the tail veins of nude mice to establish experimental lung metastases. After 42 days of continuous treatment, the number and frequency of lung metastases in doxycycline-treated animals significantly exceeded results in the control group ($P = 0.008$ and 0.04, respectively; Fig. 6*D*) and their average size was clearly larger in doxycycline-treated mice (data not shown).

Discussion

The establishment of prostate cancer metastases involves the successful negotiation of multiple endogenous physiologic barriers, survival during transit through the blood or lymphatic stream, and colonization at distant sites. The growth and metastasis of prostate cancer and other tumors is dependent on the induction of new blood vessels from preexisting ones through angiogenesis (25, 26). Cav-1 has been implicated in the regulation of endothelial cells proliferation, differentiation, and stabilization (6, 17, 27, 28). In a study using Lewis lung carcinoma cells animal cancer model, cav-1 was found to be antiangiogenic factor (29). In contrast, the results of a number of studies including this report have shown a proangiogenic function for cav-1. In an experimental melanoma model, impairment of pathologic angiogenesis was reported in *cav-1*$^{-/-}$ compared with *cav-1*$^{+/+}$ (30). Increased expression of cav-1 and microvessel density was found to be associated with metastasis and a worse prognosis in human clear cell renal cell carcinoma, suggesting a proangiogenic role for cav-1 (31). We also presented correlative evidence for a proangiogenic role of cav-1 in human prostate cancer (4). Endogenous levels of cav-1 expression in endothelial cells may provide an explanation for this controversy. *Cav-1*$^{-/-}$ endothelial cells showed abrogated tubule formation and reduced NO production with or without VEGF treatment. Enforced expression of relatively low levels of cav-1 in cav-1$^{-/-}$ endothelial cells produced increased eNOS phosphorylation (S1177) and NO production in response to VEGF treatment, yet expression of higher levels of cav-1 blocked this process (17).

Apparently, without cav-1, endothelial cells do not undergo proper maturation and maintain a hyperproliferative state. This

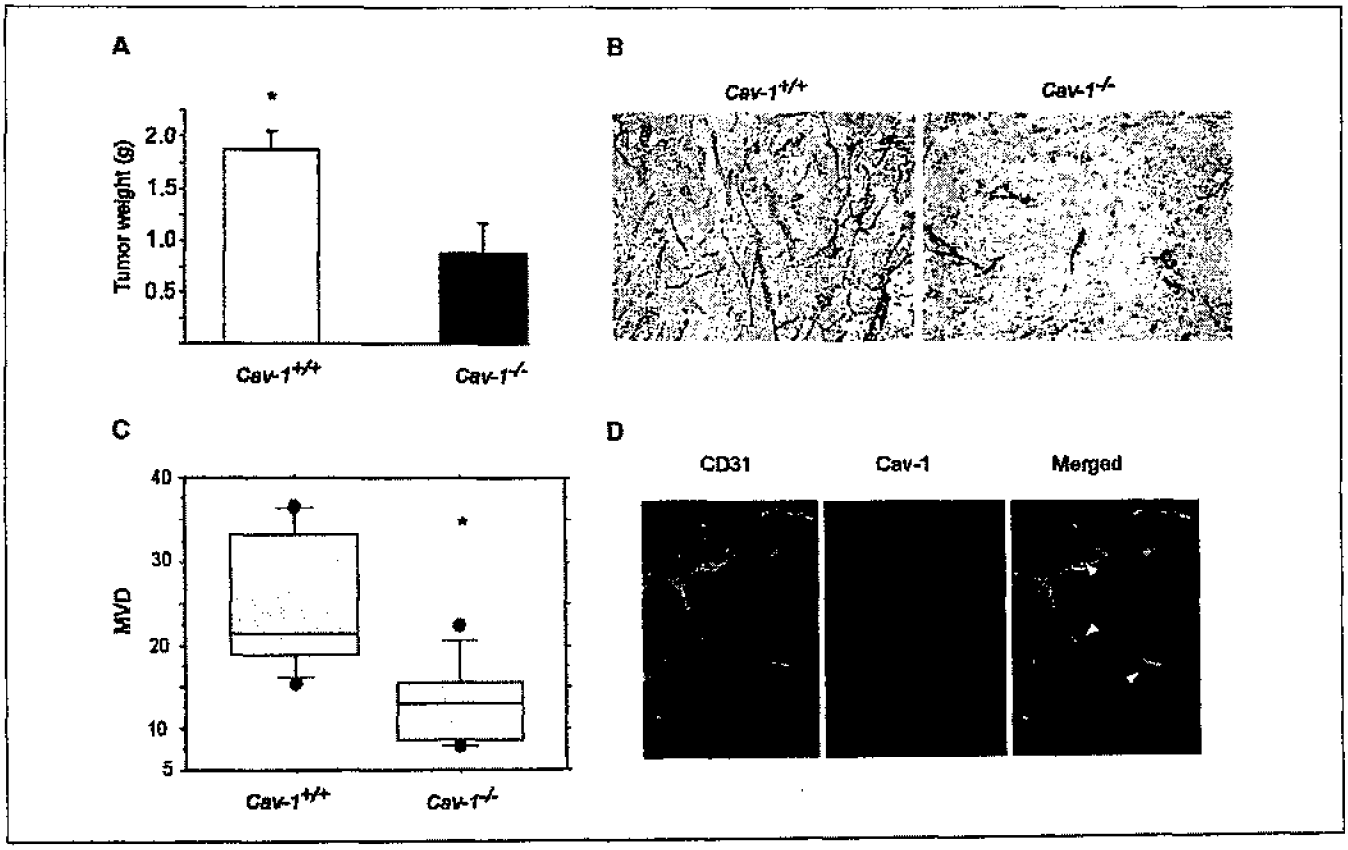

Figure 5. Secreted cav-1 promotes growth and angiogenesis in orthotopic RM-9 mouse prostate cancer model. *A*, increased RM-9 tumor wet weight in *cav-1*$^{+/+}$ hosts ($n = 7$) compared with *cav-1*$^{-/-}$ hosts ($n = 7$). *Columns*, mean; *bars*, SE. *, $P = 0.045$ by two-sided *t* test. *B*, immunohistochemical staining for CD31 in RM-9 tumors shows increased microvessel density in *cav-1*$^{+/+}$ hosts compared with *cav-1*$^{-/-}$ hosts. *C*, quantitative box plot analysis of the microvessel density (*MVD*) in RM-9 tumors from *cav-1*$^{+/+}$ versus *cav-1*$^{-/-}$ hosts. *Top lines*, 10th percentile; *bottom lines*, 90th percentile; *middle lines*, median value. *, $P = 0.0078$ by Mann-Whitney rank test. test. *D*, images of double immunostaining for CD31 (*green*) and cav-1 (*red*) in a tissue section of an RM-9 tumor from a *cav-1*$^{-/-}$ host. Arrows in the merged image (*yellow*) indicate the uptake by microvessels of cav-1 secreted by RM-9 tumors.

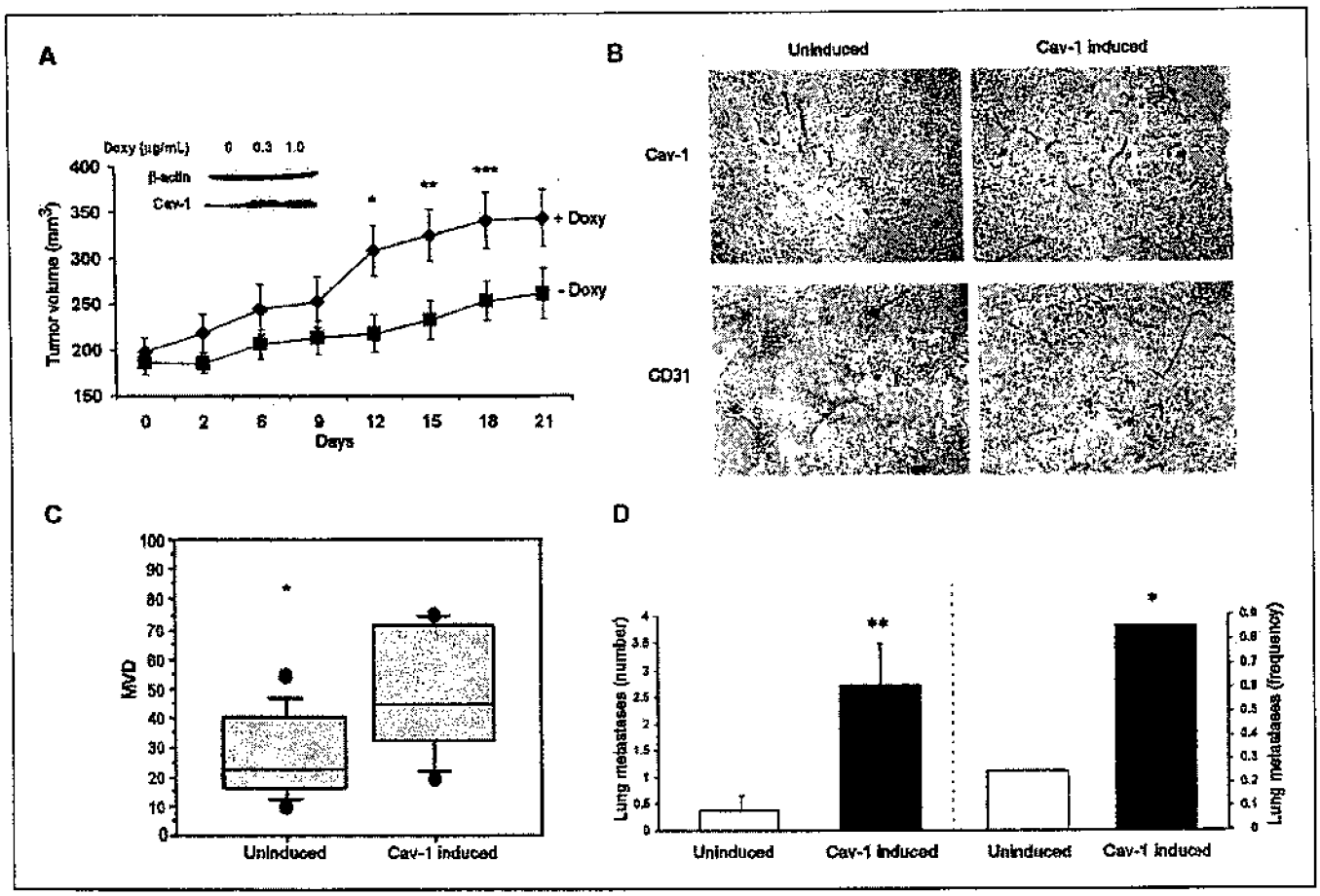

Figure 6. Secreted cav-1 promotes growth and angiogenesis in LNTB25cav tumors. *A*, Cav-1 induction by doxycycline (*Doxy*) leads to increased tumor volume in LNTB25cav s.c xenograft tumors growing s.c. Two groups of mice ($n = 8$ each) normalized for tumor volume were treated with either doxycycline (2 mg/mL) or control sucrose in drinking water for 21 d. *Points*, mean; *bars*, SE. *, $P = 0.01$ 95; **, $P = 0.035$; ***, $P = 0.019$ by two-sided *t* test. *B*, representative immunohistochemical staining for cav-1 and CD31 shows increased cytoplasmic cav-1 in cancer cells (*top*), and increased numbers of microvessels (*bottom*) in cav-1–induced LNTB25cav tumors compared with uninduced LNTB25cav tumors. *C*, quantitative box plot analysis of microvessel density in cav-1–induced ($n = 8$) and uninduced ($n = 11$) tumors. *Top lines*, 10th percentile; *bottom lines*, 90th percentiles; *middle lines*, median value. *, $P = 0.039$ by Mann-Whitney rank test. *D*, increased number and frequency of lung metastases in cav-1–induced compared with uninduced tumors. Lung metastases were established by injecting LNTB25cav cells into the tail veins of nude mice that were subsequently treated with doxycycline ($n = 7$) or sucrose ($n = 8$) in drinking water for 42 d. *Columns*, mean; *bars*, SE. *, $P = 0.040$ by Fisher's exact test; **, $P = 0.008$ by two-sided *t* test.

leads to a lack of polarization and a failure to form intercellular junctions (32), which may compromise selective transport mechanisms for specific macromolecules. Similarly, in tumor-associated endothelial cells a certain basal level of cav-1 may be required for minimal functional capacity. We have recently shown that cav-1 low/negative endothelial cells are relevant to prostate cancer. We reported significant reduction in the density of cav-1 positive microvessels in cav-1–negative human prostate cancer tissue compared with benign prostate tissues, clarifying the existence and possible significance of cav-1–negative microvessels in these malignancies (4).

We show that endocytosis of extracellular rcav-1 occurs in cancer cells (TSU-Pr1, DU145, and PC-3) and endothelial cells (HUVEC, *cav-1*$^{-/-}$ endothelial cells, and *cav-1*$^{+/+}$ endothelial cells), and that endothelial cells take up rcav-1 through lipid rafts/caveolae and clathrin-dependent pathways. Our results also show that rcav-1 uptake does not have an absolute cellular requirement for caveolae. The involvement of multiple endocytic pathways is not unique to cav-1 internalization, as these mechanisms have been described for the internalization of a number of proteins such as protein-specific membrane antigen (33), insulin growth factor binding protein-3 (34), transforming growth factor β receptor (35), and decorin (36). A possible explanation for the internalization of cav-1 through multiple pathways is its ability to interact with and bind to a large number of signaling proteins including multiple membrane receptors (15), which places it in proximity to endosome-forming activities of various pathways.

We show that CSD is necessary but may not be sufficient for cav-1 uptake, which leads to tubule formation, cell migration, and NO production in *cav-1*$^{-/-}$ endothelial cells. These data are supported by the results of a study that identified a highly conserved region of the engrailed homeoproteins that bears a high degree of homology with the CSD and are responsible for oligopeptide or oligonucleotide transmembrane, and cellular transport (37). The CSD was also found to have the ability to direct endogeneous cav-1 to cell membranes (16). Q3

We show that cav-1 angiogenic activities involve the PI3-K-Akt-eNOS pathway but not Erk1/2. Indeed, rcav-1 treatment increases phosphorylation of Akt (S473 and T308) and, hence, eNOS phosphorylation (S1177 but not T495), leading to NO production.

Because previous studies show that Akt phosphorylates eNOS on the S1177 site, leading to eNOS activation, our results are consistent with a straight forward molecular pathway through which cav-1 uptake activates Akt, which in turn activates eNOS. However, Akt inhibitor studies indicated that Akt signaling is not the only pathway culminating in eNOS phosphorylation on S1177. That is, rcav-1–stimulated Akt activation was accompanied by inhibition of PP2A, a specific serine/threonine kinase that dephosphorylates S473 and T308 on Akt, and S1177 and T495 on eNOS (13, 21, 38). It is of interest that rcav-1 did not inhibit PP1, a serine/threonine kinase whose substrate specificity is similar to that of PP2A. Because PP1 may have selective activity for the T495 site on eNOS, which unlike the S1177 site leads to inhibition of eNOS activity, the absence of cav-1–mediated inhibition of PP1 could further contribute to eNOS activation (21). This notion is supported by the absence of increased phosphorylation of T495 on eNOS in response to rcav-1 (Fig. 4*A*, *right*). Because we previously showed that cav-1–stimulated PP1, and PP2A inhibition is mediated through direct interaction between the cav-1 CSD and PP1/PP2A binding sites in prostate cancer cells, (13) it seems reasonable to suggest that this specific interaction also applies to rcav-1–mediated inhibition of PP2A in *cav-1*$^{-/-}$ endothelial cells.

Studies with two complementary animal model systems (i.e., the RM-9-*cav-1*$^{-/-}$ host orthotopic model and the LNTB25cav xenograft model) substantiate our *in vitro* findings that tumor-associated endothelial cells internalize tumor-secreted cav-1, which is associated with tumor growth, and that overexpression of cav-1 in prostate cancer cells promotes angiogenesis and tumor growth.

Overall, our data show that prostate cancer cell–derived and prostate cancer cell–secreted cav-1 has autocrine (tumor cell uptake) and paracrine (tumor-associated endothelial cells uptake) activities that can contribute to angiogenesis, tumor progression, and metastasis. We propose that prostate cancer and potentially other malignancies that overexpress and secrete cav-1, may benefit from anti-cav-1 therapy that could involve cav-1 antibodies or peptide inhibitors of CSD.

Acknowledgments

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Elevated Expression of Caveolin Is Associated with Prostate and Breast Cancer[1]

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ABSTRACT

To identify genes associated with prostate cancer progression, we developed a strategy involving the use of differential display-PCR with a panel of genetically matched primary tumor- and metastasis-derived mouse prostate cancer cell lines. We isolated a cDNA fragment with homology to the mouse *caveolin-1* gene. Northern blotting with this fragment revealed increased caveolin expression in metastasis-derived cell lines relative to primary tumor-derived cell lines. Western blotting with a polyclonal caveolin antibody confirmed increased caveolin protein in metastasis-derived mouse cell lines and expression in three of four human prostate cancer cell lines. Immunohistochemical analysis of a human prostate cancer cell line demonstrated a prominent granular pattern of caveolin accumulation. Subsequent analysis of mouse and human prostate specimens revealed minimal caveolin expression in normal epithelium with abundant staining of smooth muscle and endothelium. The frequency of caveolin-positive cells was increased in prostate cancer with markedly increased accumulation of caveolin and a granular staining pattern in lymph node metastatic deposits. In human breast cancer specimens, increased caveolin staining was detected in intraductal and infiltrating ductal carcinoma as well as nodal disease. Caveolin therefore appears to be associated with human prostate cancer progression and is also present in primary and metastatic human breast cancer.

INTRODUCTION

Prostate and breast cancers are the most commonly diagnosed cancers in men and women, respectively (1), and are similar in that they arise in hormonally regulated secretory tissues. The ability to treat both cancers relates to the stage of the disease, with those cancers that are metastatic having a much poorer prognosis. To better understand the molecular pathways associated with metastasis, we developed a strategy to isolate genes related to metastasis by using DD-PCR[3] to compare mRNA expression in a panel of genetically matched cell lines derived from primary and metastatic mouse prostate tumors. The malignant tissues were produced using the MPR model system, which involves the induction of metastatic prostate cancer *in vivo* by the transduction of the *ras* and *myc* oncogenes in fetal prostate tissues from p53 knock-out mice (2). Multiple sets of clonal cell lines from both primary and metastatic tumor foci recovered from the same inbred, experimental animal were established and analyzed for differential gene expression using a modified DD-PCR protocol (3, 4). Subsequent screening of mRNAs derived from these panels of matched primary and metastatic cell lines served to further resolve the validity of the DD-PCR results. One of the cDNA fragments identified using this approach encoded a portion of caveolin. Caveolin is a major structural protein of caveolae, lipid-based organelles that are part of the trans-Golgi network and involved in many cellular processes, such as signal transduction and transport of small molecules into cells (5–7). Formation of a multimeric complex involving glycosylphosphatidylinositol-linked uPAR, integrins, and caveolin has been shown to correlate with uPAR-mediated extracellular matrix adhesion in 293 cells (8). We analyzed caveolin expression in both mouse and human prostate cancer cell lines by Western blotting and confirmed elevated levels in metastasis-derived cells. Immunohistochemical studies demonstrated a granular staining pattern specific for caveolin in a high percentage of metastatic human prostate cancer specimens and in primary and metastatic human breast cancer. Overall, our results establish a significant association between caveolin expression and prostate cancer progression. In addition, increased accumulation of caveolin was demonstrated in primary and metastatic breast cancer relative to normal epithelium.

MATERIALS AND METHODS

Cell Lines. Mouse prostate cancer cell lines were derived from primary tumors (PA or PB) or metastatic deposits (LMA, LMB, LMC, LMD, LM1, LM2, and MMA) in the same host animal implanted with a *ras* + *myc* initiated p53 nullizygous MPR (2). The cell lines were analyzed for retroviral integration

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[3] The abbreviations used are: DD-PCR, differential display-PCR; MPR, mouse prostate reconstitution; uPAR, urokinase plasminogen activator receptor; dNTP, deoxynucleotide triphosphate.

pattern by Southern blotting (2) and cultured as described previously (2, 9). All murine cell lines were used at passages 7–10.

Human prostate cancer cell lines LNCaP, DU145, and PC-3 were obtained from the American Type Culture Collection and cultured as described previously (10). The human prostate cancer cell line ND-1 (11) was cultured in DMEM with 10% fetal bovine serum.

RNA Isolation and Northern Blot Analysis. RNA was isolated from cell lines as described previously (2) or with commercially available RNA isolation reagents (Biotex). mRNA was purified from total RNA with PolyATtract mRNA Isolation System (Promega Corp.).

For Northern blot analysis, 20 μg of total RNA were fractionated under denaturing condition on a 1% agarose, 6.7% formaldehyde gel and transferred onto Hybond-N Nylon membrane (Amersham). The membrane was baked at 80°C for 2 h. Blots were prehybridized for 2 h at 65°C in 7.5% SDS, 0.5 M sodium phosphate buffer (pH 7.2), 1 mM EDTA, 4× Denhardt's solution (50× = 1% Ficoll, 1% polyvinylpyrrolidone, and 1% BSA), and 50 μg/ml salmon testes DNA. Hybridization was carried out by adding a ^{32}P-labeled probe that had been purified with a QIA quick spin column (QIAGEN) and incubating overnight at 65°C. Blots were washed at 65°C for 20 min with 40 mM sodium phosphate (pH 7.2), 5% SDS, followed by another wash at 65°C for 20 min with 40 mM sodium phosphate buffer (pH 7.2), 1% SDS.

DD-PCR. One (primer 3, TCTGCGATCC) of a set of unique 10-mer deoxyoligonucleotide primers with an arbitrary sequence was used for reverse transcription and as both a 5′ and 3′ primer for amplification by PCR. The primers were selected based on having approximately the same ratio of G + C to A + T with no uninterrupted self-complementary of more than two nucleotides (12). Reverse transcription of mRNA was with the Perkin-Elmer Cetus GeneAmp RNA PCR kit. A reaction volume of 10 μl contained 5 mM $MgCl_2$, 1× PCR buffer II, 1 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), 1 unit/μl RNase inhibitor, 2.5 units/μl reverse transcriptase, 250 ng of primer, and 60 ng of mRNA. The reaction mixture was covered with 50 μl of mineral oil and incubated at 22°C for 10 min and 42°C for 15 min and terminated by incubation at 99°C for 5 min. The reaction was immediately diluted to 50 μl and adjusted such that it contained 2 mM $MgCl_2$, 1× PCR buffer II, 1.25 units of AmpliTaq DNA polymerase, and 20 μCi of [^{33}P]dATP (3000 Ci/mM). No additional dNTPs or primers were added so that the final concentration was 0.2 mM of each dNTP and 250 ng of primer. The PCR consisted of 40 cycles at 94°C for 40 s, 40°C for 2 min, and 72°C for 35 s with a final extension period of 72°C for 4 min.

Samples from the PCR were separated on a nondenaturing 5% polyacrylamide gel (29:1) with 5% glycerol at 9 W for 18 h. The gel was transferred to Whatman 3MM paper, dried, and exposed to X-ray film overnight. The differentially displayed bands were excised from the dried polyacrylamide gel and soaked in 500 μl of H_2O for 15 min at room temperature to remove the filter paper, and the gel slice was transferred to 20 μl of TE buffer, smashed, and incubated at room temperature 2 h to overnight. A 5-μl aliquot was reamplified in a 50-μl PCR mixture containing 1× PCR buffer II, 2 mM $MgCl_2$, 0.25 mM dNTP, 1.25 units of AmpliTaq DNA polymerase, and 1 μg of primer. The PCR was 45 cycles with the same parameters as above. The reamplified cDNA fragments were purified on 2% NuSieve agarose (FMC) by gel electrophoresis. The bands were excised and used to make a ^{32}P-labeled probe for Northern blot analysis as described above or cloned into a TA cloning vector (pCR II vector from Invitrogen). The cloned DD-PCR fragment was sequenced with Sequenase version 2.0 (USB).

Protein Isolation and Western Blot Analysis. All cell lines were grown to subconfluence and lysed with Laemmli sample buffer [125 mM Tris-HCl (pH 6.8), 2% SDS, 5% glycerol, and 1% 2-mercaptoethanol]. Lysates were separated on a 12.5% polyacrylamide-SDS gel and electrophoretically transferred to a nitrocellulose membrane. The membrane was reacted with a polyclonal caveolin antibody (C13630; Transduction Labs) at 1:2000 or a monoclonal β-actin antibody (A5441; Sigma) at 1:5000. The immunoblots were stained, using an avidin-biotin-complex (ABC) kit (Vector). Preabsorption of caveolin antiserum with specific antigen in excess resulted in a negative reaction against a positive control human endothelial lysate (not shown). This caveolin antiserum was raised against a 97-amino acid NH_2-terminal fragment of human caveolin-1. The mouse caveolin-1 protein is 96.9% identical to human caveolin-1 in this region, whereas the human caveolin-2 protein is 15.8% identical, although this region does contain an eight-amino acid conserved domain shared by caveolin-1 and -2 (13). Because of the possibility that this polyclonal antibody could recognize caveolin-2 as well as caveolin-1 protein, we also performed Western blotting with a rabbit polyclonal antibody specific for amino acids 2–21 of human caveolin-1 (sc-894; Santa Cruz Biotechnology) and a caveolin-2 specific monoclonal (C57820, Transduction Labs). Although caveolin-2 was detected in most specimens, the results confirmed that caveolin-1 was the predominant species expressed in the mouse and human cell lines (not shown).

Human Tissues Used for Immunolocalization of Caveolin. Formalin-fixed, paraffin-embedded prostatic tissues including normal epithelium (13 cases), hyperplastic epithelium (17 cases), and primary adenocarcinomas (46 cases) were used. Prostate tissue was obtained from radical prostatectomy specimens. All prostate cancers were staged using the American Joint Committee on Cancer Tumor-Node-Metastasis classification (14) as either $T_1/T_{2a}N_0$ (n = 29) or T_3N_1 (n = 17), and all tissues were examined and assigned a grade by a single pathologist (T. M. W.). For the $T_1/T_{2a}N_0$ patients, clinical follow-up was for at least 5 years, and patients with increased prostate-specific antigen level to >0.4 ng/ml were assumed to have a clinical recurrence. In addition, 25 lymph node metastatic deposits (of which, 8 were derived from the set of 17 T_3N_1 patients used in this study) were also examined. The breast tissues included benign epithelium (24 cases), intraductal carcinoma (15 cases), invasive ductal carcinoma (15 cases), and lymph node metastasis (9 cases) and were obtained from the Pathology Department of The Methodist Hospital (Houston, TX).

Immunohistochemistry. Formalin-fixed, paraffin-embedded mouse and human tissue sections were reacted with a polyclonal antiserum against caveolin (C13630; Transduction Labs) at 1:400 dilution and visualized with the ABC detection system (Vector Labs). Control incubations were done using either normal rabbit

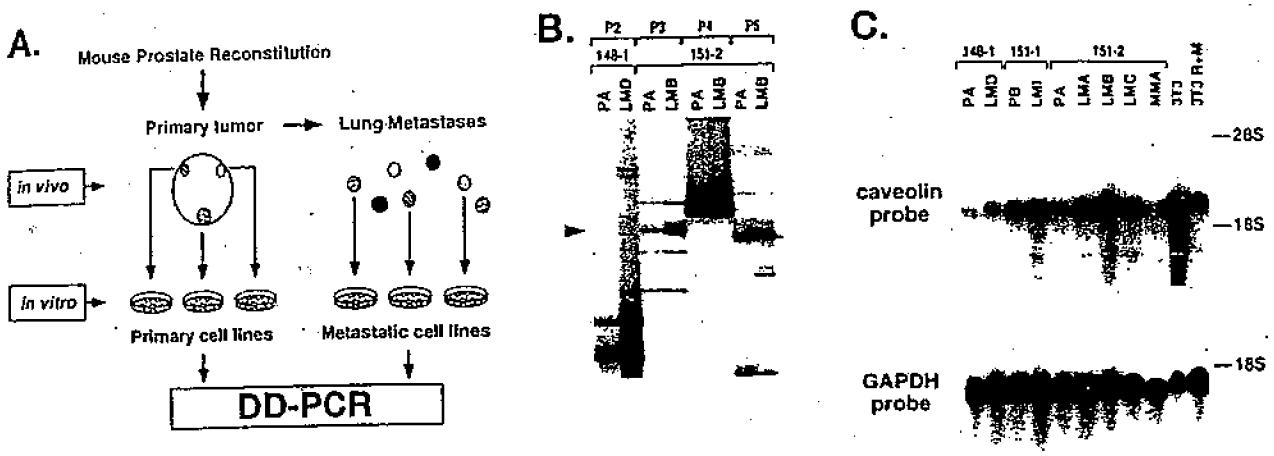

Fig. 1 *A*, diagrammatic scheme. In the MPR system, primary tumors and metastases from the same animal are explanted, and cell lines are generated. mRNA is isolated from each cell line and used for DD-PCR. *B*, a portion of a gel comparing two different, matched primary and metastatic cell lines with four different primers (P2–P5). A differentially expressed band in 151-2 LMB with P3 was subsequently identified as caveolin. *C*, a Northern blot probed with the caveolin probe or a glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probe depicting overexpression of caveolin mRNA in metastatic cell lines compared with primary cell lines and down-regulation in NIH 3T3 cells infected with a *ras* + *myc* retrovirus (2).

serum or PBS in place of the primary antibody or caveolin antiserum preabsorbed with purified caveolin peptide (Transduction Labs) in excess. All control experiments resulted in negative immunoreactivity. Positive immunoreactivity in the prostate and breast cancer specimens was defined as greater than one measuring field (at ×200) showing a granular immunostaining reaction. All slides were scored in a blinded fashion by two independent observers (G. Y. and L. D. T.). In addition to the staining with the C13630 antibody, independently obtained sections from 14 of the 46 human prostate cancer specimens (10 from the T_1/T_2 and 4 from the T_3 group) and adjacent sections from the complete set of 15 intraductal and 15 infiltrating ductal breast carcinomas specimens as well as two of the lymph node metastases that were stained with the polyclonal caveolin antiserum (C13630; Transductions Labs) were also reacted with the caveolin-1-specific polyclonal antibody (sc-894; Santa Cruz Biotechnology). The results were the same with one exception, a positive prostate cancer specimen converted to negative. In addition, subsets of breast and prostate cancer specimens were also stained with a caveolin-2-specific monoclonal antibody (C57820; Transduction Labs). Staining of cancer cells was negative, yet abundant endothelial staining was observed (data not shown).

Statistical Methods. For analysis of caveolin expression in human prostate and breast cancer, the Fisher's Exact test was used to compare the frequency of caveolin-positive specimens.

RESULTS

MPR Differential Display Cloning System for Metastasis-related Genes: Identification of Caveolin. To investigate the genetic basis of metastasis, we developed a strategy to compare and isolate the species of mRNA expressed by genetically matched pairs of primary and metastatic mouse prostate cancer cell lines, using DD-PCR. These cell lines have been characterized and shown to demonstrate differential responses to specific growth factors such as transforming growth factor-β1 (9) as well as differential metastatic activities *in vivo* (15). Because these cells have identical genetic background, originate from primary and metastatic tumors from the same animal, are of low passage, and have been derived in a similar fashion, we used these cells to demonstrate differential gene activities and isolate corresponding cDNA fragments for further analysis (Fig. 1*A*). One of the fragments detected as differentially expressed in the initial comparison is shown in Fig. 1*B*. Using primer 3, increased levels of this fragment were detected in 151-2 LMB, a clonal cell line derived from a lung metastasis, relative to that detected in 151-2 PA, a cell line derived from the primary MPR tumor in the same animal. This fragment was isolated and cloned, and 230 bp were sequenced; upon comparison with GenBank version 86.0, the sequence was similar at the nucleotide level to human caveolin-1 cDNA (16). Subsequent comparison with the National Center for Biotechnology Information BLAST WWW server revealed that the 5′ end of the DD-PCR fragment was 100% identical over 75 bases of the 3′ end of the mouse *caveolin-1* gene (17) and 85% identical over 82 bases of the human *caveolin-1* gene but was not similar to the human *caveolin-2* gene (13).

The cloned DD-PCR fragment was used for Northern blotting analysis to screen an extended panel of primary and metastatic cell lines derived from three different animals, and in every case increased steady-state levels of caveolin mRNA were demonstrated for the metastasis derived cell lines relative to their matched primary cell lines (Fig. 1*C*). Interestingly, in a comparison of NIH 3T3 cells with polyclonal Zipras/myc 9-infected NIH 3T3 cells, caveolin mRNA levels were reduced in the transformed cells relative to their nontransformed and nontumorigenic partner, in general agreement with results obtained previously using NIH 3T3 cells with single oncogenes including *ras* (18).

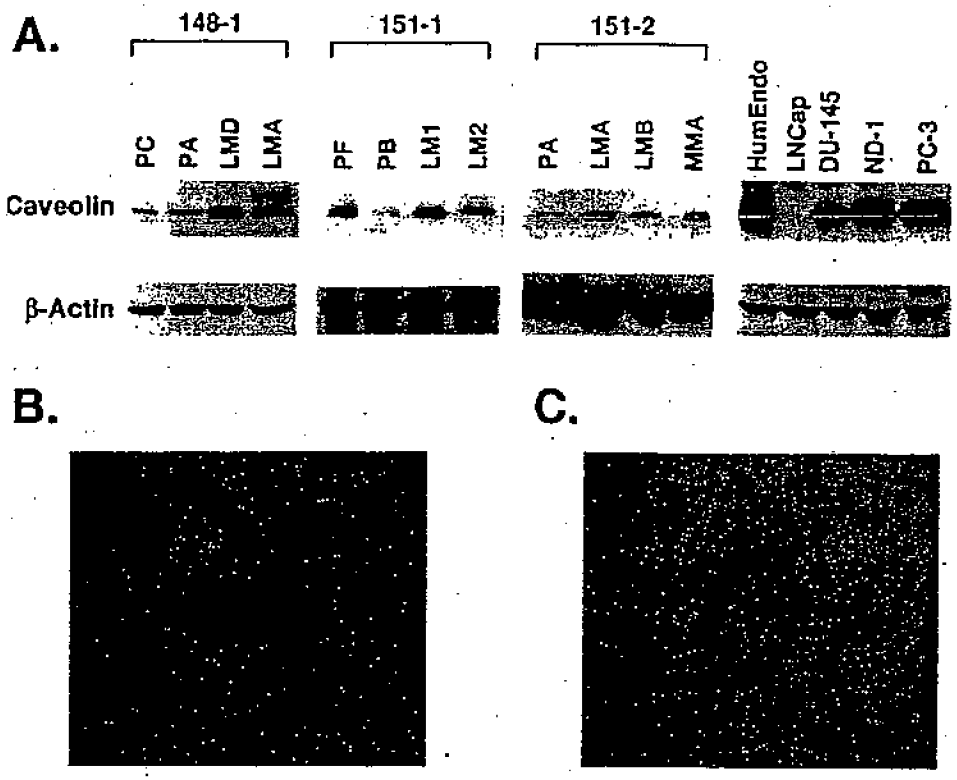

Fig. 2 *A*, expression of caveolin protein relative to β-actin in primary MPR tumor-derived and metastatic site-derived cell lines from three experimental mice (148-1, 151-1, and 151-2) and human prostate cancer cell lines. A protein extract from human endothelial cells (Hum Endo; Transduction Labs) was used as a positive control. *B*, caveolin immunohistochemistry of ND-1 cells grown *in vitro* with punctate staining pattern apparent in some cells; ×400. *C*, caveolin immunohistochemistry of ND-1 cells grown *in vitro* also revealed areas of cytoplasmic staining that were often localized at point of attachment to the substrate. ×200.

Elevated Caveolin Protein Levels in Mouse and Human Prostate Cancer Cell Lines Derived from Metastases. To evaluate expression of caveolin at the protein level, pairs of primary as well as metastatic cell lines from three different animals (148-1, 151-1, and 151-2) were compared by Western blotting using a commercially obtained antibody to caveolin. The results (Fig. 2*A*) demonstrate an overall 2–3-fold increase of caveolin protein in metastatic-derived cell lines relative to their matched primary cell line counterparts. To extend these results to human prostate cancer, similar Western blotting experiments were performed on four human prostate cancer cell lines and a human endothelial cell line (Hum Endo), as a positive control. Three of four of the human prostate cancer cell lines were derived from metastases, and one (ND-1) was derived from a high Gleason grade primary carcinoma. In three of four cases, caveolin protein was abundant (Fig. 2*A*). The exception was metastasis-derived, androgen-sensitive LNCaP cells. The pattern of localization of caveolin within human prostate cancer cells *in vitro* was evaluated by immunohistochemistry. In some cells (*e.g.*, ND-1 cells), caveolin was detected within the cytoplasm of cells in a granular pattern (Fig. 2*B*). Interestingly, in many cases accumulation of caveolin appeared to be localized to substrate attachment sites (Fig. 2*C*).

Association of Caveolin Expression with Tumor Progression Using Immunohistochemical Staining of Prostate and Breast Cancer Specimens. To validate the *in vitro* studies using cell lines, a series of immunohistochemical studies were undertaken to assess the pattern and amount of caveolin expression in tissue specimens of both primary and metastatic prostate carcinoma. Initially, specimens derived from normal mouse prostate and primary and metastatic mouse prostate carcinoma generated by the MPR model were analyzed. The results demonstrated only minimal caveolin expression in normal mouse prostate epithelial cells within the prostate gland; however, abundant caveolin staining was observed in smooth muscle cells, which uniformly surround mouse prostate acini as well as endothelial cells in the stromal compartment (Fig. 3*A*). A diffuse, increased accumulation of caveolin was seen in primary prostate cancer (Fig. 3*B*), and in the corresponding metastatic cancer cells within the mesentery, higher levels of caveolin appearing as a granular pattern localized near the plasma membrane were seen (Fig. 3*C*). In normal human prostate, as in the mouse, accumulation was seen in smooth muscle cells as well as endothelial cells with minimal or no staining of ductal or acinar epithelial cells (Fig. 3*D*). In primary prostate cancer, detectable accumulation of caveolin in malignant cells was occasionally observed (Fig. 3*E*), whereas in metastatic cancer within lymph nodes, an obvious granular accumulation of caveolin was seen in the carcinoma cells (Fig. 3*F*).

A semiquantitative scoring system for caveolin staining based on the frequency of caveolin-positive cells was applied to areas of normal human prostate, benign prostatic hyperplasia, and primary and metastatic prostate carcinomas (Table 1). The results indicated a very low frequency (7.7%) of positivity in cases of normal glandular epithelium and low but increased frequency of caveolin-positive epithelium in cases of hyperplasia (17.6%) and primary cancer of low stage ($T_1/T_{2a}N_0$; 13.8%).

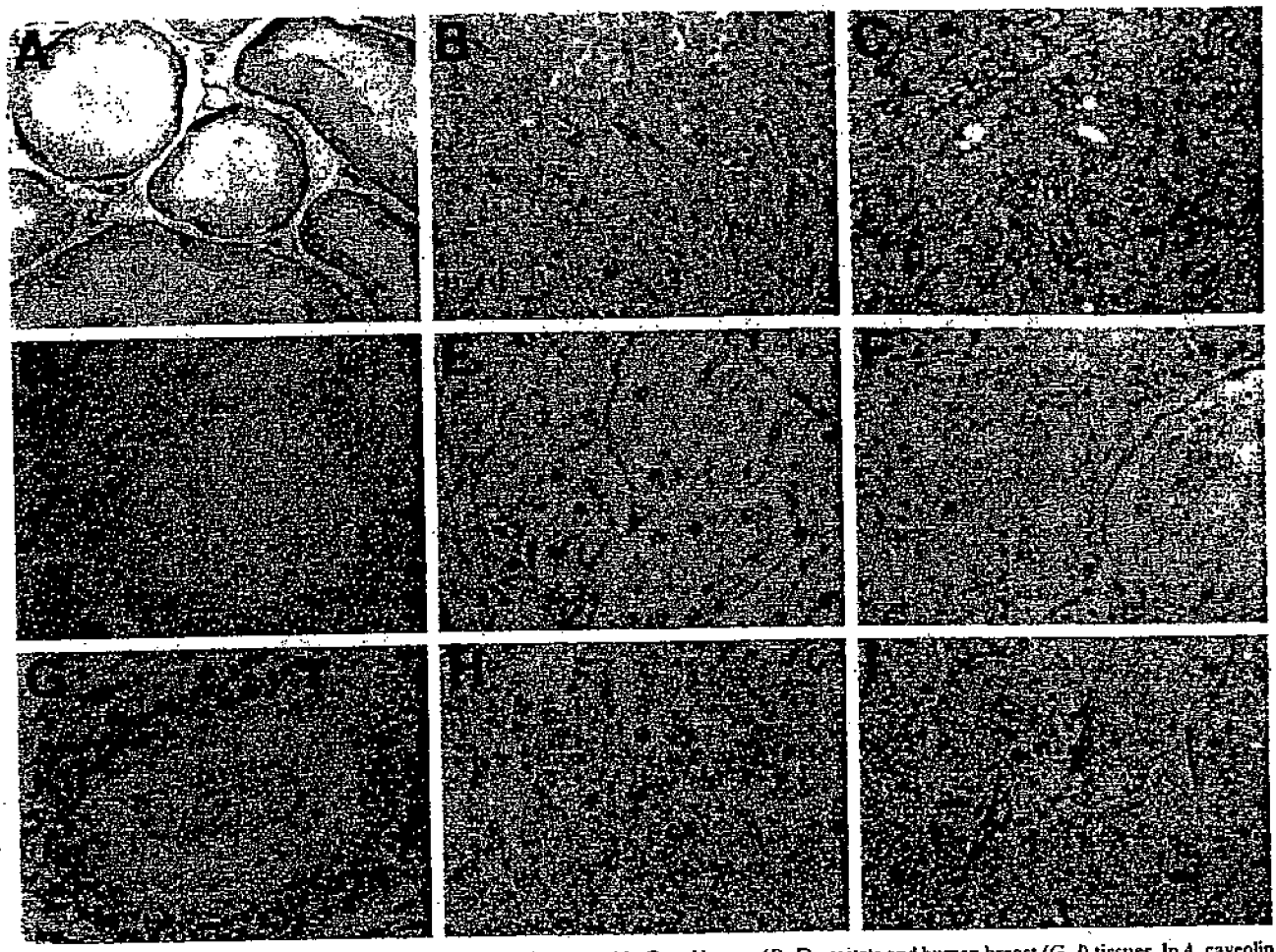

Fig. 3 Immunostaining with a polyclonal caveolin antibody in mouse (*A–C*) and human (*D–F*) prostate and human breast (*G–I*) tissues. In *A*, caveolin reactivity was confined to the smooth muscles immediately adjacent to glandular epithelium of normal mouse prostate. In *B*, punctate immunoreaction product appeared in primary mouse prostate cancer. Increased immunoreactivity localized adjacent to the cell membrane, was present in cancer cells located in the mesentery (*C*) of the same animal as in *B*. In normal human prostate, caveolin reactivity was localized in the stroma. Most primary human prostate (*E*) cancers revealed weak or no (*D*) caveolin immunoreactivity compared with the granular immunoreaction products seen frequently in metastatic cancer cells located in lymph nodes (*F*). Caveolin was detected in the myoepithelial cells of normal human breast tissue and not detected in the luminal epithelial cells (*G*). Prominent granular immunoreaction products accumulated in cancer cells of intraductal carcinoma (*H*) and infiltrating ductal carcinoma (*I*).

The $T_1/T_{2a}N_0$ patients were subdivided into those who remained cancer free for 5 years after radical prostatectomy and those whose prostate cancer recurred. No statistical difference was seen between the recurrent and nonrecurrent group. Although no association was found between caveolin and prostate cancer recurrence, our sample size was small, which limited our ability to detect any difference. Thus, our lack of a difference may reflect the limited statistical power of our test rather than a true lack of association. Increased frequency of caveolin staining was seen in T_3N_1 primary cancers of high stage and with nodal metastasis (T_3N_1; 29.4%), and markedly increased levels of caveolin staining were detected in cancer cells metastatic to lymph nodes (56% $P < 0.01$; Fisher's Exact test).

To determine whether this phenomenon was shared by other hormone-sensitive adenocarcinomas in humans, a series of breast carcinomas and nonneoplastic breast tissues was also evaluated for caveolin expression using the same staining technique. As in prostate, caveolin staining in normal ductal or lobular epithelial cells was minimal, whereas prominent caveolin staining was observed in the adjacent myoepithelial cells (Fig. 3*G*). However, intraductal carcinomas stained positive with a similar granular pattern as that observed in metastatic prostate cancer, yet more striking (Fig. 3*H*). As in prostate cancer, increased levels of caveolin staining were detected in breast cancer cells metastatic to lymph nodes (Fig. 3*I*). Careful quantitative analysis using the described scoring system confirmed that significantly higher expression of caveolin was detected in intraductal carcinomas relative to normal breast epithelium ($P < 0.001$; Fisher's Exact test) and a statistically significant increase was demonstrated for infiltrating ductal carcinoma as well as nodal metastases ($P < 0.001$; Fisher's Exact test for both comparisons; Table 2).

Table 1 Caveolin immunostaining in human prostate tissues

Prostate specimens	n[a]	Caveolin −	Caveolin +	Positivity[b] %
Normal glandular epithelia	13	12	1	7.7
Hyperplastic epithelia	17	14	3	17.6
Pathological stage of cancers				
$T_1/T_{2a}N_0$	29	25	4	13.8
Recurrent	11	9	2	18.2
No recurrence	18	16	2	11.1
T_3N_1				
Primary cancer	17	12	5	29.4
Metastatic site in lymph node	25[c]	11	14	56.0[d]

[a] *n* values denote the number of patients in each group.

[b] Positivity was defined as over one measuring field showing granular immunostaining in cancer.

[c] Tissues from metastases included eight cases of T_3N_1 stage, for which the primary cancer was also stained.

[d] $P < 0.01$ (Fisher's Exact test) as compared with both normal and hyperplastic epithelia as well as the T_1/T_{2a} cancers.

Table 2 Caveolin immunostaining in human breast tissues

Breast specimens	n	Caveolin −	Caveolin +	Positivity %
Benign epithelia	24	22	2	8.3
Intraductal carcinoma	15	3	12	80.0[a]
Infiltrating ductal carcinoma	15	1	14	93.3[a]
Lymph node metastasis	9	2	7	77.8[a]

[a] These values are significantly higher than that in the benign breast epithelia ($P < 0.001$; Fisher's Exact test).

DISCUSSION

We have previously established sets of early-passage cell lines from primary and metastatic mouse prostate cancer that were initiated in the same animal by transduction of *ras* and *myc* oncogenes into fetal prostate tissues from p53 knock-out mice, *i.e.*, MPRs (2). These sets of cell lines are clonally tagged by the initiating retrovirus, are early passage, and are genetically and biologically matched, such that the predominant genetic differences between the primary *versus* metastasis-derived cell lines should be related to the metastatic process. Using this system together with DD-PCR techniques, we identified the *caveolin* gene as being up-regulated in both mouse and human prostate cancer metastases and interestingly in primary and metastatic breast cancer.

Previous studies of caveolin function in normal cells have revealed its involvement in many biological activities that are germane to cancer progression. Caveolin is a major protein constituent of caveolae, a recognized subcompartment of the plasma membrane and Golgi network (19). Caveolae are strategically positioned to sequester glycosylphosphatidylinositol-linked proteins and apparently organize their interaction with downstream cytoplasmic signal transduction complexes (5, 6, 20). Caveolae identified in nonmalignant cells play important roles in signal transduction (20–22), molecular transport (23), and cellular motility and adhesion (8). In regard to signal transduction, specific molecules involved in transformation have been associated with caveolae including members of the *ras* family (24), c-*src* (25, 26), as well as the endothelin receptor (21). Although the specific roles for these molecules in prostate and breast cancer progression remain to be fully elucidated, mutations or aberrant expression of these molecules have been identified in these malignancies (27–29). Caveolae are also involved in the molecular transport of ceramide and cholesterol. Because ceramide has been clearly demonstrated to be involved with apoptotic activities, inappropriate transport of this molecule could perturb the apoptotic pathway and play a role in cancer progression (30). Recently, it was demonstrated that caveolin mRNA levels are up-regulated by free cholesterol in human skin fibroblasts (31), and increased expression of caveolin-1 has been reported in both mouse (32) and human (33) cells with impaired ability to metabolize low density lipoprotein-derived cholesterol. Interestingly, a prospective study of dietary fat and risk of prostate cancer reached the conclusion that advanced prostate cancer was associated with high fat intake, especially fat derived from red meat (34). In addition, caveolin, together with β-1 integrin and uPAR, was identified as components of a functional complex involved in matrix attachment and motility (8), two biological activities that are highly relevant to the metastatic cascade. Although further studies are necessary to dissect the discrete functions in which caveolin participates, recent studies suggest that caveolin may be involved with bridging integrin-mediated signaling with Shc and further downstream signal transduction pathways that result in gene activities that are relevant to the metastatic cascade (8, 35, 36).

Although caveolin is involved in numerous biological activities relevant to malignant progression, direct evidence of a role for caveolin in progression of human carcinoma has not been reported. A recent study demonstrated that overexpression of selected dominantly acting oncogenes resulted in suppression of caveolin mRNA and protein levels and fewer caveolae in fibroblastic NIH3T3 cells (18). In this report, it was further shown that caveolin levels were inversely correlated with the size but not the number of colonies produced in soft agar by oncogene-transfected clones. In our study, transformation of NIH3T3 cells with both *ras* and *myc* also led to reduced caveolin mRNA (Fig. 1*C*). The down-regulation of caveolin in transformed NIH3T3 cells appears to contradict the major observations of our present study; however, this may only reflect differences in transformation and selection of immortalized fibroblasts *in vitro* compared with the malignant progression of cancer cells within prostate or breast tissue *in vivo*. Previous studies have demonstrated that caveolin expression is associated with the differentiated phenotype in some simple squamous epithelia including capillary endothelial cells, type I pneumocytes, and specific mesenchymal cells, including fibroblasts, smooth muscle cells, and adipocytes (37–41). Our analysis demonstrates that caveolin is barely detectable in normal prostate and breast glandular epithelium *in vivo*, whereas adjacent smooth muscle, endothelial, and breast myoepithelial cells had abundant staining.

The results of this study clearly associate increased accumulation of caveolin with progression of human prostate cancer and with primary and metastatic breast cancer relative to normal epithelium. Prostate cancer cell lines derived from high-grade localized disease (ND-1), as well as metastases, expressed high

levels of caveolin *in vitro*. Further analysis using immunohistochemistry indicated extensive accumulation in metastatic disease. These results were closely mimicked in breast cancer tissues. However, our analyses also demonstrated widespread expression of caveolin in localized breast disease, suggesting that increased caveolin expression occurs earlier in progression of breast cancer relative to prostatic cancer. Alternatively, metastasis without extensive local growth may occur more frequently in caveolin-positive prostate cancer relative to caveolin-positive breast cancer. Regardless of the timing of elevated caveolin during the progression of these important malignancies, metastatic lesions of both prostate and breast cancer are characterized by significantly increased levels of this protein. Further studies should reveal additional information regarding a potential role of caveolin in prostate and breast cancer progression.

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